Adolescent Cannabis Use and Brain Structure in Young Adulthood

by

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Abstract

Cannabis is the most commonly used illicit substance in the world. Grey matter cortical thickness in young adulthood was compared in a sample of over 500 male participants from the ALSPAC prospective longitudinal cohort study based their cannabis use status (Never, Early User, Late User) at age 21, and their cumulative cannabis use at age 16.5 (Golding, Pembrey, & Jones, 2001). Thickness did not differ between early late and never users, however among early users those with the largest cumulative use had a thinner cortex relative to those who used the least after controlling for biological, environmental, behavioural, and substance use confounders in a GLM. Using CB1 receptor gene expression data from the online Allen Human Brain Atlas (restricted to the left hemisphere), we determined the heaviest users exhibited a thinner cortex in regions with the highest and lowest densities of CB1 receptors; psychosocial, pharmacological, and hormonal mechanisms could potentially explain our findings (Hawrylycz et al., 2012).

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Introduction

1 Introduction

1.1 Epidemiology

Cannabis is the most common illicit substance used today, with annual prevalence (in 2014) reaching 180.6 million among people aged 15-64 years (World Drug Report: United Nations Office on Drugs and Crime, 2014). Globally, annual prevalence of cannabis use increased from approximately 2.5% to 3.9% between 2009 and 2013 (UNODC, 2013). The highest annual prevalence rates of cannabis use belong to West and Central Africa (12.4%), Australia and New Zealand (10.9%), North America (10.7%), and West and Central Europe (7.6%), all surpassing the global average. The largest rise in prevalence rates over the years has been seen in the Americas (UNODC, 2014). In the United States, surveys show cannabis is believed to be the least harmful of illicit drugs, and the UN has linked lowered perceived risk of cannabis to an increase in its use (UNODC, 2014). According to a study by National Institute on Drug Abuse (NIDA), 60% of senior high-school students perceived regular consumption of cannabis not to be harmful - in contrast to only 30% holding this opinion 20 years ago (Johnston, O'Malley, Miech, Bachman, & Schulenberg, 2014). The 2010 Annual report on the state of the drugs problem in Europe (EMCDDA), a survey of 30 European countries, estimated lifetime prevalence of cannabis use is 22.5%. Thus, approximately 75.5 million (22.5%) Europeans between the ages of 15 and 64 have used cannabis, with 23 million (6.8%) having used cannabis in the last year, and 12.5 million (3.7%) having used cannabis in the last month (EMCDDA, 2010; 2014). The average lifetime prevalence of cannabis use ranged from 10% to 30% of the population, while the total range of cannabis use across all countries varied widely, from 1.5% to 38.6% (EMCDDA, 2010). Lifetime cannabis use in the United Kingdom – where the sample studied here originates - was higher than the European average, estimated at 31.1% (EMCDDA, 2010).

1.1.1 Burden of disorder

Between 2008 and 2009, prevalence of lifetime cannabis abuse and dependence in the U.S. were 3.9% and 8.3%, respectively; these numbers are based on a sample of 15,500 participants of the National Longitudinal Study of Adolescent Health (Haberstick et al., 2014). Between 2006 and 2010, the number of people in the US seeking treatment for cannabis use disorders increased by 14%, and the number of cannabis-related hospital visits increased by 56%. It has been suggested that increases in treatment for

cannabis use may be related to observed increases of cannabis potency over the years (Johnston et al., 2014; UNODC, 2014). By 2010, 13.1 million individuals around the world met criteria for cannabis dependence; of those, 1.8 million (13.4% of all cases) were in North America (Degenhardt et al., 2013). According to Degenhardt's (2013) analyses of data from the Global Burden of Disease study in 2010, prevalence of cannabis dependence peaks between 20 and 24 years. Likewise, Haberstick et al. (2014) found that lifetime dependence onset peaked at age 20 years in the United States. It is estimated that 9% of cannabis users become dependent, a risk almost doubles if cannabis use onset occurs during adolescence (Wagner and Anthony, 2002). An increased risk of dependence based on adolescent onset is concerning given recent trends showing that prevalence of cannabis use is increasing among adolescent populations (Wagner & Anthony, 2002).

1.1.2 Age differences

Overall, cannabis use is more prevalent during adolescence and young adulthood relative to later life periods. In 2010, annual cannabis prevalence was 14.1% in adults, as compared with 24.5% among high-school students in the United States (World Drug Report: United Nations Office on Drugs and Crime, 2010). The European School Survey Project on Alcohol and Other Drugs (ESPAD) reported average lifetime prevalence of cannabis use in high-school students throughout Europe to be 17% - more than double that of other illicit drugs (2011 ESPAD Report). Adolescents and young adults between 15 and 24 years of age exhibited the highest rates of cannabis use in 29 of the 30 European countries surveyed in the 2010 EMCDDA. Estimated lifetime cannabis use was higher in the adolescent and young adults aged 15 to 24 (30.9%) relative to the general population between 15 and 64 years of age (22.5%), while use in last year (16%) and month (8.4%) were almost double that of older adults, with rates of 6.8% and 3.7%, respectively. According to the 2011 Canadian Alcohol and Drug Use Monitoring Study (CADUMS), which surveyed over 10,000 Canadians, rates of past year cannabis use were three times higher in youth (21.6%) relative to use by adults (6.7%). In an annual study monitoring substance use trends among secondary school students in the United States, NIDA reported approximately 7% of students smoked cannabis almost daily (Johnston , O'Malley, Bachman, & Schulenberg, 2012). Of most relevance to our study are rates of lifetime cannabis use in the United Kingdom, where annual prevalence (use in the last year) was similarly higher among youth between 15 and 24 years of age (40.1%) as compared with all (15 to 64 years) individuals (30.2%) according to the EMCDDA (2010). Greater cannabis and other drug use exhibited by younger populations is speculatively attributed to social trends, specifically of adults having a lower propensity towards breaking laws and social norms, supported by comparisons of age distribution patterns of adult use of legal recreational psychoactive drugs (2012 World Drug Report); this explanation possibly explains why the upwards trend of substance use during adolescence and young adulthood does not continue later in life.

Prevalence among adolescents and young adults also appears to be increasing. In 2010, 13% of European high-school students reported use within the last 12 months as compared with only 8% in 1995 (2011 ESPAD Report). Increases in annual prevalence rates among high-school students were reported in 11 of the 36 European countries surveyed. France for example observed an increase in reports of cannabis use in the previous 30 days by almost 10% in 4 years - from 14.9% in 2007 to 23.8% in 2011; this increase for cannabis was high relative to that of other substances such as alcohol, which only increased by 0.6% over the same period (2011 ESPAD Report).

1.1.3 Sex differences

Both regular and problematic cannabis use were more prevalent among male relative to female high-school students. Sex differences were evident in the majority of European countries participating in ESPAD, with boys having used cannabis more often than females in 27 of the 36 countries surveyed, typically by 6 percentage points (2011 ESPAD Report). On average, lifetime cannabis use rates were 19% in male students, as compared with 14% in female students across European countries (2011 ESPAD Report). Males were 1.5 times more likely to endorse cannabis use within the last month as compared with females (2011 ESPAD). Compared with their female peers, male students between the age of 15 and 16 years were more than twice as likely to have used cannabis more than 40 times in their lives (2011 ESPAD Report). Based on findings from the 2014 European Drug Report, this trend appears to persist in adulthood; 75% of Europeans between the age of 15 and 34 years who smoke cannabis daily or almost daily (20 days or more in a month) are male (EMDCCA, 2014). Males also make up 83% of cannabis users who enter treatment (EMDCCA, 2014). In young adults, the male to female ratio of using cannabis use in the last year ranged from 6.4: 1 in Portugal to 1.4: 1 in Bulgaria (EMCDDA, 2010). In the United States, individuals over the age of 12 years were more likely to have ever used cannabis if they were male (9.7%) as compared with female (5.6%) according to the 2013 National Survey on Drug Use and Health (Substance Abuse and Mental Health Services Administration, 2014). In Canada, the 2011 CADUMS study by Health Canada report shows that cannabis use is twice as common in males (12.2%) than in females (6.2%) over 15 years of age. In the United Kingdom, lifetime cannabis use was more frequent among adult males (36.3%) than their female (24.3%) counterparts, as well as among young adult males between the ages of 15 to 24 years (44.6 %) relative to their young adult female counterparts (24.3%).

1.2 Rational

1.2.1 Need for research

Need for Research Taken together, the above research suggests there is a great need for a clear understanding of cannabis' effects on brain health given the current trends in cannabis use, and further

that this need will become greater in the near future, given potential increases in cannabis use upon its legalization. A study of high school seniors (n = 6116) in the U.S. reported that – if made legal - 10% of non-cannabis using students intended to use cannabis while 18% of students with lifetime cannabis use reported intentions to use cannabis with greater frequency (Palamar, Ompad, & Petkova, 2014). For this age group, a 10% increase in lifetime users would correspondingly increase lifetime prevalence by 5.6%, from 45.6% to 51.2% among high school senior students (Palamar et al., 2014). In light of higher prevalence rates and increased vulnerability for dependence, the above reports suggest special attention should be paid to adolescents and, in particular, males to increase our understanding of both the consequences of cannabis use and its individual-based predictors. The fact that cannabis' potency is increasing, in stride with its popularity, compounds concerns. There is a growing body of evidence suggesting that cannabis-related risks, such as dependence and psychosis (one of several psychopathologies that has been attributed to cannabis use) are dose-dependent (Degenhardt et al., 2013). Insights into differential effects of dose and potency would prove particularly valuable in confirming the hypothesized relationships. Elucidation of cannabis' neural "footprint" may also yield clinical applications useful in the treatment of the increasing burden of cannabis dependence. Finally, adding to the urgency for this research is the changing social climate towards cannabis as not harmful, and political climate towards cannabis legalization (Johnston , O'Malley, Bachman, & Schulenberg., 2013; Palamar et al., 2014). Cannabis' title as an "illicit" substance is currently in question, before the question of its safety has been answered.

1.2.2 Design of current study

Adolescence and young adulthood are, respectively, the periods of cannabis-use initiation and its peak use (Degenhardt et al, 2013; Haberstick et al., 2014). During the first of the two periods, important developmental changes are taking place in both the body and the brain as a part of sexual maturation. For example, structural changes in the brain have been observed during puberty; volumes of white and grey matter increase and decrease, respectively, during this period (reviewed in Giedd et al., 1999; Giedd, 2004; Paus, Keshavan, & Giedd, 2008). These age-related changes in brain morphology are, in part, related to changes in the hormonal environment of the adolescent brain; for example, the male brain is exposed to an increase in testosterone over the course of puberty. We hypothesize that normal maturation processes taking place in the adolescent brain may render it more vulnerable to potentially noxious exposures, such as cannabis, and that these effects would be reflected in brain morphology.

The present investigation explores the relationship between cannabis use during adolescence and brain structure at the completion of adolescence in a large cohort of participants from the Avon Longitudinal Study of Parents and Children based in the United Kingdom (ALSPAC: www.alspac.bris.ac.uk; Golding, Pembrey, & Jones, 2001). This cohort has been assessed longitudinally

from birth, throughout childhood and adolescence through to the age of 21 years, and measures of substance use, psychopathological symptoms, cognitive performance are available for multiple time points during late childhood and adolescence. In addition, multimodal magnetic resonance images (MRI) of the brain were collected from a subset of male participants when they were between 18-21 years of age. A longitudinal design, with an initial time point preceding the onset of either substance use or psychopathology makes this particular data set valuable. Also of advantage to our study is the fact that substance use data have been acquired prospectively at multiple time points, allowing us to identify the onset of substance use. Thus, we are able to track participant substance use during one of the heaviest periods of illicit substance use, according to studies of prevalence rates (see above). Finally, based on research showing that males are more likely to consume cannabis as compared with females, the fact that our data set comprised of males only will likely maximize the rates of cannabis use in our sample, allowing us a greater opportunity to investigate its possible consequences. Overall, our ALSPAC sample of male youth, combined with longitudinal data, optimizes our ability to detect possible correlates of cannabis use in early adolescents on the brain of young adults.

1.3 Biology

1.3.1 Pharmacology

The primary psychoactive constituent in cannabis is Δ9-tetra-hydrocannabinol (THC), which can be introduced to the system in a variety of ways, most typically delivered through inhalation (Gaoni & Mechoulam, 1971). Due to the psychoactive effects of THC, cannabis has long been used as a recreation drug and, more recently, has been utilized in various medical applications. In the human central nervous system, THC primarily binds to cannabinoid-1 (CB1) receptors, one of two known cannabinoid receptor subtypes in the body. While CB1 is highly concentrated in the brain, the other, CB-2 receptors, are mainly present in the peripheral nervous system (Glass, Faull & Dragunow, 1997).

As a drug, cannabis appears to induce a combination of hallucinogenic, stimulant, and depressant effects (Block, Erwin, Farinpour, & Braverman, 1998). Studies attributed many of the psychological and physiological acute effects of smoking cannabis specifically to THC binding at CB1 receptor sites in the brain. In a randomized placebo-controlled study of human males, the subjective “high” in response to smoking cannabis was blocked in a dose-dependent fashion by SR141716, a CB1 receptor antagonist (Huestis et al., 2001). Paired with an observed reduction in subjective intoxication was a 59% reduction in cannabis-related tachycardia after CB1 antagonist administration (Huestis et al., 2001). More recently, Han et al., (2012) showed the abolishment of typical cognitive and neural responses to synthetic THC in *CB1* knockout mice (deleted *CB1* receptor gene). Synthetic THC is known to produce effects analogous to THC such as impairments in spatial working memory and long-term depression of synapse strength. A

synthetic THC induces these effects in normal (wild type) mice but not in *CB1* knockout mice, suggesting that at least some of cannabis' effects are orchestrated through binding to CB1 receptors. Further, some level of correspondence between localization of CB1 receptors and the subjective effects of cannabis has been noted. Brain regions exhibiting higher concentrations of CB1 receptors, such as the hippocampus and amygdaloid complex, are functionally associated with subjective effects of cannabis, such as memory deficits and mood alteration including euphoria and anxiety (Glass et al., 1997; Herkenham et al., 1990; Katona et al., 2001; Lorenzetti, Lubman, Whittle, Solowij, & Yücel, 2010). Katona et al., (2001) noted there exists exceptions to this overlap, such as low CB1 receptor densities in the nucleus accumbens (highly involved in reward processing) in the context of cannabis as a drug found to be highly rewarding and psychologically addictive. Evidence suggests more research is needed to understand the underlying relationship between CB1 receptor binding and the effects of cannabis. Overall, research points to CB1 receptor binding, its density and regional distribution, as being related to effects of cannabis, and should thus be considered upon their investigation.

1.3.2 Localization of cannabinoid-1 receptors

Glass and colleagues (1997) were the first to label and quantify cannabinoid receptors in the adult human brain. Using autoradiography, CB1 receptors were labeled with a synthetic cannabinoid agonist. After dissection and staining, computerized densitometry methods quantified receptors in the brains of eight individuals ranging in age from 21 to 88 years (mean = 55). Glass (1997) noted that binding sites were distributed heterogeneously throughout the brain. Moderate densities of CB1 receptors appear throughout the neocortex, especially in the most superficial layers (Glass et al., 1997). Concentrations were highest in the hippocampal formation, substantia nigra, globus pallidus, cerebellum, and regions of the association cortex; all those with a mean over 100 femtomoles bound/milligram of brain tissue (fmol/mg) included: hippocampus, entorhinal cortex, amygdaloid complex, middle frontal gyrus, and cingulate gyrus, right and left substantia nigra, internal globus pallidus and the molecular layer of the cerebellum (Glass et al., 1997). Two similar studies support high CB1 densities in the substantia nigra, medial globus pallidus, basal ganglia, and cerebellum, followed by the cerebral cortex and hippocampus (Biegon & Kerman, 2001; Herkenham,1990). Overall, more cannabinoid binding sites were seen in the left hemisphere as compared with the right, by about 56-150 % across the eight individuals. Particularly high concentrations within the left hemisphere were noted in language areas. Relatively higher densities were present in associational cortices in frontal, temporal, and medial temporal lobes (Glass et al., 1997). Relatively lower densities were present in the primary motor cortex and primary sensory cortical regions (Glass et al., 1997). Across the cerebral cortex, CB1 binding is highest in the superficial layer and decreases with every subsequent (deeper) layer (Glass et al., 1997; Biegon & Kerman, 2001). Neocortical regions with the highest concentrations were associational areas in frontal lobe and medial temporal lobe.

Biegon and Kerman (2001) compared CB1 expression in fetal and adult brains. Fetal brains exhibited lower concentrations of CB1 (Biegon & Kerman, 2001). For comparison, fetal mean fmol/mg were 289.2 (+/- 11.4), approximately half that of young adults 567 (+/- 175). Interestingly, receptor density increased with (gestational) age slowly, without reaching adult levels by the end of the second trimester (Biegon & Kerman, 2001). Following most of the protocol laid out by Glass, a later study found an increase in CB1 receptor densities with age when comparing fetal/neonatal, infant/child and adult brain sections (Mato, Del Olmo, & Pazos, 2003). Less regional specificity was also observed, with homogeneously low levels of CB1 present across cortical and subcortical gray matter (Biegon & Kerman, 2001). Lastly, fetal and adult brains differed in CB1 distribution; most dramatically, fetal brains exhibit much lower densities in caudate and putamen regions, in comparison with other regions, which reached adult expression levels very early (by ~17 weeks gestation), such as the globus pallidus pars medialis. Conversely, high CB1 densities appear prenatally in some regions that exhibit very low densities in adulthood (Mato et al., 2003). Altogether this suggests CB1 receptor formation and distribution continues into at least early development. In rats, CB1 receptor densities appear to peak during adolescence (Batalla et al., 2013). As CB1 levels during adolescence have not yet been measured in humans, whether this peak occurs in humans is currently unknown.

Localization of regions with high densities of cannabinoid receptors can also be inferred from the expression of the cannabinoid-receptor-1 (*CNR1*) gene. The Allen Brain Institute (ABI) quantified expression of all genes in the adult human brain and mapped it to all structures across the entire brain; this dataset is freely available online (at www.brain-map.org). The ABI Human Brain Atlas was compiled based on over 500 tissue samples, per hemisphere, from 6 brains of donors with no known history of psychiatric or neurological conditions; 4 donors provided left hemisphere only, 2 donors provided both hemispheres. Over 60,000 gene probes were used in the creation of each genome-wide expression profile.

Broadly, we synthesized microarray gene expression data from the ABI and averaged *CNR1* expression in the cerebral cortex. Across donors expression of CNR1 was consistent, correlating highly. Across the cortex expression was somewhat uniform, with the hippocampal, olfactory, and orbitofrontal regions exhibiting higher values. Found below, Table 1 shows the mean expression of the 10 brain regions with the highest bilateral *CNR1* gene expression, which ranged from ~4.89 to ~7.57 pmol/mL. As pointed out above, gene expression was measured in both hemispheres for only 2 of the 6 donors; in comparing mean gene expression levels in the same regions between hemispheres in these two donors, the left-hemisphere regions showed greater expression than the right-hemisphere regions most of the time, and there were more regions in the left side as compared with the right side falling in the top quintile of brain regions ranked by mean expression. These findings are consistent with the autoradiography data reviewed above.

Table 1. Brain regions exhibiting the highest *CB1* gene expression.

Region	CB-1 Gene Expression
CA2 field	7.57
Piriform cortex	7.38
Hippocampal formation	6.66
Parolfactory gyri	6.65
Anterior orbital gyrus	6.45
Paraterminal gyrus	6.40
Gyrus rectus	6.36
Temporal pole	6.33
Superior rostral gyrus	6.32
Short insular gyri	6.31

1.4 Literature Review

To elucidate the effects of smoking cannabis on human brain structure, previous studies have used magnetic resonance imaging (MRI) to compare grey and white matter properties between either cannabis users and non users, or heavy and light cannabis users. These studies have yielded inconsistent findings. Discrepancy among findings may in part be attributable to discrepancy in design across studies, as well as small sample sizes in some cases (and, in turn, the possibility for false negative and false positive findings). Despite mixed results in the literature, a majority of the literature regarding grey matter points to a negative association between cannabis use and the "amount" of grey matter

1.4.1 Grey matter studies

Differences in grey matter based on cannabis user status have been reported by numerous studies. One of the earliest studies to report differences in brain morphology related to cannabis smoking was in 2000 by Wilson and colleagues, who found lower relative volume of grey matter and higher relative volume of white matter volume (% of brain volume) in adults (n = 57; mean age = 31.3; range = 19 - 48 yrs) with an earlier onset of cannabis smoking (before 17 yrs) as compared with those with a later onset (after age 17 yrs). A smaller whole brain volume was also associated with early cannabis use (Wilson et al., 2000). Matochik and colleagues (2005) observed a similar relationship in young adults (mean age = 25.4 yrs) using voxel based morphometry (VBM), an automated and therefore non-biased statistical method used to compare differences on a voxel-by-voxel basis across the whole brain or within

predetermined regions of interest across groups. When compared with non-users (n = 8), heavy cannabis users (n - 11) who smoked an average of 35 joints per week and had used cannabis for an average of 8 years (ranging from 2 to 22) had less grey matter (Matochik et al., 2005). Compared with non-users, cannabis users exhibited lower grey-matter density in a region of the right parahippocampal gyrus, but greater density in the right thalamus and regions near the precentral gyrus bilaterally (Matochik et al., 2005). Cannabis use onset was not a focus of Matochik et al.'s study (2005), though they reported mean onset of cannabis use in users to be 15.7 years, making the majority of participants early onset users. This is of note given that early onset use but not late onset use correlated with structural grey and white differences in cannabis users (Arnone et al., 2009; Wilson et al., 2000). Mata and colleagues (2010) conducted a cross-sectional study comparing (adult) cannabis users (n = 30) with non-users (n = 44) recruited from the general population; they reported thinner cortex in frontal regions of the right hemisphere in cannabis users. Additionally, cortical thickness correlated negatively with age in non users, cortical thickness was not related to age in cannabis users. Mata and colleagues interpreted their findings as indicative that cannabis may advance the onset of cortical changes typically associated with age. Lopez Larson et al. (2011) tested for a thinner cortex in adolescents with heavy cannabis use (n = 18) and non users (n = 18) who were young adults between 17 and 18 years old. After covarying for age and sex, clusterwise whole-brain analysis showed that cannabis users had lower cortical thickness in the insula and superior frontal regions in both hemispheres relative to non users, as well as caudal middle frontal regions in the right hemisphere; interestingly, frontal regions correlated negatively with urinary cannabinoid measures, and age of onset correlated negatively with thickness in the superior frontal gyrus in the right hemisphere. Heavy cannabis users also exhibited thicker cortex in lingual regions bilaterally, superior temporal and inferior parietal regions in the right hemisphere, and paracentral regions in the left hemisphere; lingual regions were also found to correlate negatively with cannabinoids present in urine (Lopez-Larson et al., 2011). Cousjin et al. (2012) utilized VBM to compare structural properties of numerous brain regions between heavy cannabis users (n = 33) and non-users (n = 42), and evaluated structural brain properties in relation to both current and lifetime cannabis use. Once more, lower grey matter was found in heavy cannabis users (compared with non-users), evident in negative correlations between cannabis use and dependence with volume of the amygdala and hippocampus. In contrast, regions in the cerebellum were observed to have larger volumes in heavy cannabis users as compared with non-users. Batalla (2014) performed VBM at the whole brain level and at region of interest level for four ROIs (the prefrontal cortex, neostriatum (caudate and putamen), ACC and the hippocampus-amygdala complex), revealing higher gray-matter volume in the left postcentral gyrus in cannabis users (n=29) compared with non-users (n=28). Most recently, Battistella and colleagues (2014) reported lower gray matter in cortical regions as well as greater grey matter in cerebellar regions of regular cannabis users (n=26) as compared with occasional users (n=31) matched for duration of use. Using VBM,

Battistella et al.. (2014) showed less relative grey matter within the medial temporal cortex, temporal pole, parahippocampal gyrus, insula, and orbitofrontal cortex of regular (vs. occasional) users. Further, measures of frequency of use in the prior three months and age of onset related to differences in cortical grey matter but not cerebellar differences; higher frequency and earlier age of onset were associated with lower volumes of cortical grey matter. In contrast, Gilman et al. (2014) also used VBM to compare cannabis users (n=20) and non-users (n=20) and found greater grey-matter density in cannabis users relative to non-users in a number of regions, including the left nucleus accumbens extending to subcallosal cortex, hypothalamus, sublenticular extended amygdala, and left amygdala.

In addition to studies comparing structural measures of the whole brain, there have also been several studies comparing a specific cortical or subcortical structure hypothesized to be affected by cannabis. Memory deficits associated with cannabis use led Demirakca and colleagues (2011) to compare hippocampal grey matter concentrations and volumes between chronic recreational cannabis users (n = 11) and non-users (n = 13). According to VBM analyses, cannabis users exhibited lower grey-matter volume in the right anterior hippocampus (Demirakca et al., 2011). Solowij et al. (2013) followed up previous observations of the lower hippocampal volumes in cannabis users vs. non-users and conducted shape analyses in which they compared the hippocampus in schizophrenia patients (n=17) and healthy controls (n=31), as well as between cannabis users and non-users in each group (47% of patients; 48% of healthy controls; Cousjin 2012). In the cannabis users, hippocampal "deflation" with an anterior predisposition was observed in both groups relative to non-users. Cannabis use and schizophrenia appear to contribute additively to this phenomenon in that both cannabis-use patterns and schizophrenic symptoms correlated with observed hippocampal deflation, and that the greatest difference was found between control non-users and patients who used cannabis. Churchwell, Lopez-Larson, and YurgelunTodd (2010) compared medial orbital prefrontal cortex (moPFC) volumes, of interest based on its key role in substance abuse and impulsivity, in cannabis abusing (n = 18) adolescents (16 to 19 yrs) and non-user (n = 18). The moPFC was smaller in cannabis abusing adolescents as compared with controls; the volume correlated positively with age of first use. Sex differences were found in a comparison of amygdala volumes in adolescent (16 to 19 yrs) chronic cannabis users (n = 35) and non-users (n = 47), with female (but not male) cannabis users showing *larger* volumes after covarying head size, alcohol, nicotine and other substance use (McQueeny et al., 2011).

There are studies that have failed to observe grey-matter differences between cannabis users with non-users. Shortly after Wilson and colleagues' (2000) first published evidence of structural differences in humans, Block and colleagues (2000) study reported no differences in whole brain volume of young adults (mean = 22.5 yrs), or regional white matter or grey matter among young adults who were current (and frequent) cannabis users (n= 18) and non-users (n = 13); the only reported difference was lower volumes of ventricular CSF. Importantly, Block's study used a control group of non-users; the Wilson's

study compared cannabis users who smoked different degrees (light vs. heavy). To address these contradictory results, Tzilos compared adult (mean age = 33.5 yrs) non-users (n=26) with long-term users (n=22) who all reported being over a certain threshold of lifetime use; the group mean of the latter group was 20,100 lifetime episodes of smoking. In this study, no group differences were found in the volumes of whole brain, grey matter, white matter or the hippocampal volume

1.4.2 White matter studies

Group differences between cannabis users and non-users in various measures of white matter have also been evaluated. Matochik compared frequent cannabis users (n = 10) with non-users (n = 10) and, in the users (vs. non-users) observed lower white-matter density in the left the parietal lobe and higher white-matter density in the left parahippocampal and fusiform gyri. Moreover, years of cannabis use correlated positively with white-matter density in the precentral gyrus (Matochik et al., 2005). Arnone et al., (2008) reported that heavy cannabis users with early onset (n = 11) had higher values of mean diffusivity (but similar fractional anisotropy) in the prefrontal sub-region of the corpus callosum, as compared with non-users (n = 11). A study by Ashtari et al. in 2009 used tractography-based analyses to measure mean diffusivity and fractional anisotropy, revealing that young adult males in treatment for cannabis dependence (n = 14) showed altered patterns of white matter properties in fronto-temporal connection, as compared with non-users (n = 14) (Ashtari, Cervellione, Cottone, Ardekani, &, 2009). Specifically, users (vs. non-users) showed lower fractional anisotropy, greater radial diffusivity, as well as greater trace (a measure of overall diffusivity) in the superior and middle temporal gyri, posterior internal capsule/thalamic radiations. A DTI study by Yucel et al. (2010) also reported white matter abnormalities in adolescent cannabis users (n = 11) relative to non-users (n = 8), finding lower fractional anisotropy in the fasciculus beside the right hippocampus; FA was not higher in cannabis users (vs. non-users) in any brain regions. Both lower and higher FA in adolescents (16 to 19 yrs) with concurrent use of both cannabis and alcohol (n = 36) relative to non-using controls (n = 36) were reported by Bava et al. (2010); voxelwise analyses showed users had lower FA in superior longitudinal fasciculus, postcentral gyrus, bilateral crus cerebri, and inferior frontal and temporal white matter tracts, and higher FA in occipital regions, internal capsule, and superior longitudinal fasciculus. In their comparison of cannabis users in both patients with schizophrenia (n = 17) and healthy controls (n = 31), Solowij et al. (2011) found no differences in grey matter but did find those in white matter of the cerebellum. Cannabis use was associated with lower volumes of the cerebellar white-matter in both patients and control groups. Healthy cannabis users exhibited 23.9% lower volumes relative to healthy non-users; this difference was even higher in patients, with 29.7% lower volumes than healthy non-users. Additionally, the cerebellar volume negatively correlated with duration of cannabis use in healthy cannabis users (Solowij et al., 2011). Gruber, Silveri, Dahlgren, and Yurgelun-Todd (2011) reported differences in white-matter properties

between chronic adolescent cannabis users (n=15) and non-users (n=15) using DTI; they compared measures of fractional anisotropy and diffusivity obtained in six brain regions. In comparison with non-users, cannabis users demonstrated lower fractional anisotropy in frontal regions in the left hemisphere and higher diffusivity in the genu. Additionally, age of onset (M= 14.9 yrs) correlated positively with fractional anisotropy and negatively with diffusivity. In a follow up study, Gruber et al., (2014) found that age of onset was related to white matter differences observed in chronic, heavy cannabis users (n = 25) in comparison with non-users (n = 18). Heavy cannabis users demonstrated lower FA as compared with non-users; earlier onset (before 16 yrs) cannabis users demonstrated lower FA as compared with late onset (after 16 yrs) cannabis users. Further evidenced that cannabis may lead to white-matter abnormalities was provided by Zalesky and colleagues, with comparisons of fractional anisotropy and diffusivity between long-term, heavy cannabis users (n = 59) and non-users (n = 33). Tractography analysis determined two localized (connected) networks to have smaller fibre bundles as measured by streamline (fibre bundle trajectory-based mapping) analysis: streamlines appeared reduced by 84% in the right fimbria (crus of the fornix) and by 88% in the commissural fibre running from the splenium of the corpus callosum to within the right precuneus. Regression analysis revealed that the only predictive factor of total number of tract fibers in these networks was cannabis use (e.g. not alcohol, tobacco, depression). Radial and axial diffusivity, measures indicative of lesser myelination and white matter insult respectively, correlated with age of regular cannabis use onset; other cannabis measures such as cumulative dose did not however correlate. These results are indicative of higher diffusivity (and thus less white matter integrity) in cannabis users with a later onset of regular use, evidencing cannabis use onset as determinant in brain outcomes (Zalesky et al., 2012).

A couple of studies were unable to find group differences in white matter between cannabis users and non-users. The Cousjin and colleagues' (2012) VBM study discussed above found no association between white-matter densities and cannabis use or dependence. Delisi et al. (2006) used DTI to compare white-matter properties between adolescents who were frequent cannabis users (n = 10) and non-users (n = 10). Across multiple measures no group differences were found to suggest either loss of white matter integrity or cerebral atrophy, including FA and MD measures that have shown differences in a number of studies.

1.4.3 Heterogeneity across studies

Not all studies of cannabis use and structural brain properties have reported differences between users and non-users, and there is inconsistency among those that do in terms of both the location and direction of the difference. Even among studies finding structural differences in frontal and parietal regions lobes lack replication regarding the specific region where differences were found (Churchwell et

al, 2010; Gruber et al, 2011; Lopez-Larson et al., 2011; Mata et al., 2010; Matochik et al, 2005). Authors of the studies reviewed above as well as review papers have attributed inconsistency among results to variance in methodology among studies (Batalla et al. 2013; Battistella et al., 2014; Cousjin et al., 2012; Lorenzetti et al, 2010; Quickfall & Crawford, 2006; Rochetti et al., 2013; Yucel et al., 2010). In terms of study design, previous studies differ in sample size, statistical approach, cannabis user group, and participant characteristics.

Sample sizes have varied widely in previous studies. To illustrate, Block et al. (2000) compared 18 users with 13 nonusers and Delisi et al. (2006) compared samples as low as 10 participants per group; both were among the few studies that did not observe group differences in grey and white matter respectively. In contrast, later studies compared participant groups of much larger sizes, ranging from 30 to 59 participants per group; these studies observed group differences (Mata et al. 2010; Zalesky et al. 2012). With both the cannabis group and control group combined, the smallest sample size studied was n = 19 (Lorenzetti et al., 2010) while the largest was n = 92 (Rochetti et al., 2013).

The statistical approach taken has also differed between studies. Some studies conducted analyses at the whole-brain level and were thus unbiased, while others did so at the region of interest level, often focusing on individual structures such as the hippocampus and amygdala. Region of interest studies and studies of individual structures were based on *a priori* hypotheses. For example, regions of interest were determined based on either their involvement in substance dependence, being associated with cognitive functions impaired by cannabis use, or having a high density of CB1 receptors. For example, the hippocampus was a region of interest in multiple studies due to its being both rich in cannabinoid receptors as and having a key role in memory, a cognitive function often impaired by cannabis use (Battistella et al. 2014; Cousjin et al., 2012; Demirakca et al., 2011).

Differences in statistical power may therefore have contributed to inconsistent findings based on discrepancies in sample size and level of analyses. For example, Cousijn et al. (2012) found lower volume of the hippocampi to be associated with weekly cannabis use, and volume of the amygdala to be associated with cannabis dependence only at the region of interest, but not at the whole-brain level after using a family-wise error cluster-correction ($p<0.05$). It is clear that significant results have mostly been yielded from region-of-interest studies; to mitigate the potential bias of such analyses, Rocchetti et al. (2013) used a meta-analytic approach to test whether there are any consistent differences in brain structure between cannabis users and non-users. A brain measure was included in the meta-analyses if at least 3 studies endorsed it. This threshold yielded 6 brain measures: Right and Left Amygdala, Right and Left Hippocampus, intracranial volume, and whole brain volume. No differences were found in intracranial volume, whole brain volume, or the right or left sides of the amygdala or hippocampus. The amygdaloid and hippocampi volumes showed differences when considered all together; as compared with non-users, cannabis users showed significantly lower volumes of the these two temporal-lobe structures.

Group differences in hippocampi however were only significant when combined; when either hemisphere was compared alone, this difference was no longer significant. Authors calculated a measure of publication bias (bias likelihood of being published or not according to the results) for amygdala; upon statistically correcting for this bias, group differences in the amygdala were no longer significant.

Cannabis-user groups differed across studies widely in terms of the extent of use. For example, cannabis users included individuals who had ever used cannabis, current cannabis users, heavy cannabis users, individuals abusing cannabis, and individuals in treatment for cannabis-use disorders (Ashtari et al., 2009; Churchwell et al., 2010; D'Souza, Pittman, Perry, & Simen, 2009; Gruber et al., 2014; Wilson et al., 2000). As discussed by review papers, participants' duration of use, frequency of use, and onset of use varied greatly across studies, and not every study acquired each of these measures (Batalla et al., 2013; Lorenzetti et al., 2010; Rochetti et al., 2013). When reported, mean duration of cannabis use ranged from 2 years to 23 years (Lorenzetti et al., 2010; Rochetti et al., 2013). When reported, frequency of cannabis use ranged from 1 joint per month to 63 joints per week (Lorenzetti et al., 2013). Further, users also differed in terms of their cannabis-use onset. The age of onset across studies ranged from 12 to 34 years, while the mean age of onset ranged from 16 to 20 years old (Lorenzetti et al., 2010). Importantly, it is possible that these various cannabis user groups differ not only in the degree of cannabis use, but also qualitatively; different metrics of cannabis use may interact, or be related to different structural outcomes. For example, the onset of use may determine structural differences rather than frequency of use, or they may interact. Cousjin et al.'s (2012) found cannabis use frequency and dependence were both associated with different structural regions, and in different directions. Cannabis use and dependence negatively correlated with amygdala and hippocampus volumes, in contrast to frequency of cannabis use, which correlated positively with regions in the cerebellum (Cousjin et al. 2012).

Age, sex, and polydrug use also varied widely across studies (Batalla et al., 2013; Lorenzetti et al.; Rocchetti et al., 2013). More recent studies have focused on adolescent and young adult participants. The average age of participants in studies ranges from 17 to 40 years old (Batalla et al., 2013; Rocchetti et al., 2013). Like the present study, some studies included male participants only (Ashtari et al., 2011; Arnone et al. 2008), some studies included 1 female in each the user and control group (Delisi et al. 2006; Gruber et al. 2005), whereas others included larger proportions of female participants (Tzilos et al., 2005 [27%]; Cousjin et al. 2011 [37%]). Participant polydrug use also differed from study to study. For example, some studies used polydrug use as exclusion criteria (Cousjin et al. 2012). In contrast, other studies included participants with heavy use of other substances (Ashtari et al.s 2009; one third of heavy cannabis users met DSM-IV criteria for past alcohol abuse).

1.5 Summary

In light of design heterogeneity and inconsistent findings across studies discussed above, our study and analyses have been designed to eliminate some of the discussed sources of variance. Specifically, participants in the present study are all male and part of a birth cohort, therefore eliminating and minimizing variation of sex and age in our sample, respectively. Further, multiple metrics of cannabis use were obtained, including age of cannabis-use onset, early cumulative use, and frequency of use. Characteristics of cannabis use in which our participants differed were either incorporated as independent measures (e.g. cannabis use onset, cumulative number of uses by age 16) or statistically controlled for (e.g. frequency of cannabis use at age 18 to 21). Lastly, we controlled statistically for use of other common substances (cigarette smoking, binge drinking), both in early adolescence and young adulthood (i.e., at the time of scanning).

Overall, there appears to be more evidence in support of a relationship between cannabis use and structural brain properties based on a majority of studies reporting group differences between cannabis users and non-users. Although this is the case for both grey and white matter, the evidence is stronger for the former. Therefore, we have started our examinations of this sample by focusing on grey matter and hypothesized we would find group differences in cortical thickness.

Chapter 2
Methodology

2 Methodology

2.1 Sample population

The Avon Longitudinal Study of Parents and Children (ALSPAC) is an ongoing longitudinal population-based cohort, situated in the former Avon Health Authority of England (Golding, Pembrey, & Jones, 2001). Upon its commencement in the early 90s 14,541 pregnant mothers enrolled in the study, resulting in 13,988 infants one year after birth making up the total cohort, a representative sample population of the UK (Fraser et al., 2013; Boyd et al., 2013). This reflects the number of singleton and twin births one year after their birth. A large number of children's characteristics, such as cognitive abilities, mental health, and body composition, have been collected repeatedly over the past 20 years. In addition, a wealth of information about the children's family environment and genetic/epigenetic variations is available. Approval for the current study was obtained from the ALSPAC Law and Ethics Committee and the Local Research Ethics Committees. More information on the ALSPAC sample is available online (http://www.bris.ac.uk/alspac/).

This study focused on a subset of male participants (n=508) for whom magnetic resonance images (MRIs) of their brains were collected following the research goals of an NIH grant funding these acquisitions (R01 MH085772-01A1; Axon, Testosterone and Mental Health during Adolescence). In addition, levels of testosterone were assayed from blood obtained at multiple time points between 9 and 17 years of age (Khairullah et al., 2014). At 18-21 years of age (Mean ± SD: 235.45 months ±10.07 months, range: 216 to 258 months), MRIs and a set of additional measures (mental health, substance use, saliva) were collected. The selection of participant for this subset was based on their residing within 3 hours, one-way, from the scanning site, and the availability of at least three blood samples acquired between 9 and 17 years of age.

2.2 Acquired measures

2.2.1 Independent measures

Participants' exposure to cannabis was measured via self-reports across seven different time points from 10 to 21 years of age. In either an interview or questionnaire, participants answered the question if they "had ever tried cannabis?", thus enabling classification of participants as having either a positive or negative exposure status at each time point (Table 1). Those answering "Yes" answered

additional questions probing metrics characterizing individuals' experience with cannabis (e.g. number of times cannabis was used; peers' use of cannabis).

Table 2. Collection of cannabis variables, and cannabis exposure status across study time points (n = 508).

Timepoint	Participants		Method
	Age	Exposed	
T1	10	0	Focus Group Interview
T2	12.5	10	Focus Group Interview
T3	13.5	17	Focus Group Interview
T4	14	32	Child Completed Questionnaire
T5	15.5	97	Computerized Questionnaire
T6	16.5	106	Child Completed Questionnaire
T7	18 - 21	274	Questionnaire at Time of Scan

By the age of 18 to 21, 53.6% (n=274) of males reported they had smoked cannabis at least once. For the purposes of this study, we are concerned with measures of cannabis use collected when participants were approximately 16.5 years old (T6), and later at 18 and 21 years old (T7). Our independent variables were operationalized based on data collected at these time points: Cannabis Use (CU) and Cumulative Dose (CD).

Cannabis use. Based on self-report at T6 and T7, participants were categorized as belonging to one of three groups: Early Users, Late Users, and Never Users.

We defined a cannabis user as ever answering "yes" to the question "Have you ever tried cannabis (also called marijuana, hash, dope, pot, skunk, puff, grass, draw, ganja, spliff, joints, smoke, weed)?" (Golding, J. (2001). ALSPAC [Study Questionnaires]. Retrieved from http://www.bris.ac.uk/alspac/researchers/resources-available/data-details/questionnaires/). Thus, the threshold for classifying a participant as a cannabis user is: having tried marijuana at least once. Cannabis users were then classified as Early Users or Late Users based on the time point they first answered "yes" to ever having tried marijuana. Early Users were operationalized as first reporting ever having used cannabis at 16.5 yr (T6) or any previous time point. In contrast, Late Users were operationalized as first reporting ever having used cannabis at 18-21 yr (T7). Therefore, an Early User is defined as having used cannabis during adolescence by the approximate age of 16.5 yr, whereas a Late User is defined as first having used cannabis in late adolescence or young adulthood, between the approximate ages of 16.5 and 21 yr. Early Users and Late Users combined will be referred to as "Cannabis Users."

We chose 16.6 yr (T6) as the threshold for classifying participants into either Early Users or Late Users groups in order to maximize the number of individuals classified in the Early User group, while keeping with the literature in which age 16 and 17 or younger has been considered early use (Arnone et al., 2008; Batalla et al., 2014; Wilson et al., 2000). Not surprisingly, drug experimentation correlated positively with age in our sample (see Table 2.). Previous studies showed that use before and after the age of 16 differentiated outcome measures among cannabis users (Lisdahl, Gilbart, Wright, & Shollenbarger 2013; Gruber, Sagar, Dahlgren, Racine, & Lukas, 2012). Furthermore, maximizing the number of Early Users maximized the subset of participants for whom data regarding Cannabis Dose could be analyzed; this was because information about cannabis dose was not collected at the only later time point (T7).

Cumulative dose. An ordinal measure of individuals' cumulative exposures to marijuana was collected, probing participants to indicate the "number of times [they had] smoked marijuana in total." These data were quantified on a 5-point ordinal scale: [Never], [<5], [6-20], [20-60], [60-100], [100+].

Cumulative dose was captured via self-report on a questionnaire at three time points throughout the study, when participants were 14, 15.5, and 16.5 years of age. As mentioned above, this measure was unavailable for the final time point at the time of scan (T7), when participants were young adults between the ages of 18 and 21. For our analyses, we chose the most recent time point (T6) in order to maximize the sample size available for these analyses. As mentioned above, these data were not available for our Late Users group. Since cannabis dose is not applicable to our Never Users group, our analyses of cannabis dose data refers only to our Early Users participant group.

Given this observed correlation between age and cannabis use, we would expect to find fewer participants in categories with the highest cumulative dose, considering participants were only in mid-adolescence. Indeed, that is what we found; there were considerably fewer participants in the latter two categories. To reduce the discrepancy in distribution of participants between categories, the two highest-dose categories were collapsed into one: [60+]. Thus, Early Users were classified into 1 of 4 Cumulative Dose comparison groups: [<5], [6-20], [20-60], [60+] times of having had smoked marijuana in total.

2.2.2 Exclusions

Missing Data. With respect to self-report data, exclusion criteria were established for cases where participants could not be classified with confidence into groups due to either missing or inconsistent data. Regarding MRI data, participants whose MRI scans did not pass quality control standards were also excluded from the final analyses.

As is often the case with longitudinal data, data were not collected at every time point from every participant due to a variety of reasons. Reasons provided for missing data are detailed in Table 2 below. The majority of missing data was due to being "Not Completed", which represents participants who were

not invited to complete the “Child Completed Questionnaire” at T4 and T6 (i.e., data were not collected). The category “No response/Skipped” represents the only cases where participants chose not to answer, leaving only 5 participants who we could not classify on either cannabis use or cannabis dose. Considering the sensitive nature of illicit drug use, missing data due to participant omission may introduce self-report bias. But only fewer than 1% of participants in our sample could not be classified due to such omitted responses, indicating that potential self-selection bias has been minimized in our data set.

Table 3. Categorization of missing data.

	T1	T2	T3	T4	T5	T6	T7
"Did not start session"	2	2					
"No response"/"Skipped"				6	1	5	
"Did not attend"		15	9		16		
"Did not start task"	5	7	3		16		
"Not Completed"				89		108	
Blank	13						2
Total	**20**	**24**	**12**	**95**	**33**	**113**	**2**

Missing data resulted in the inability to classify some participants, resulting in their exclusion from analyses. Participants for whom data were missing for at least the last time point, who had only previously responded with “No” to ever having tried marijuana were excluded as they could not be ruled out as a Late User. Participants missing data both T5 and T6, who had only previously responded with “No” to ever having tried marijuana for all earlier time points but responded with “Yes” at T7 were excluded, as they could not be classified as being either an Early User or Late User.

Inconsistent data. A number of participants were excluded because their exposure status could not be classified with confidence due to self-contradiction in reporting. Exclusions due to inconsistent data were only applicable to our Cannabis User variable since data collected at multiple time points were involved, unlike our Cumulative Dose variable. Participants who responded with “No” to ever having tried marijuana after already reporting “Yes” at a previous time point were labeled as being inconsistent. Although most such cases were excluded, some participants labeled inconsistent were included in the final data set. Thus, inconsistent participants who reported “No” after having reported “Yes” 1 or 2 times were excluded (n = 14). But inconsistent participants who reported having tried marijuana at least 3 times were included in the final data set as Early Users (n = 3). One participant was excluded for contradictory responses in the same questionnaire, reporting both that they had tried marijuana before, and that the last time they tried marijuana was “Never” (n = 1).

QC failures. Of all 508 scanned ALSPAC participants, we could classify 484 based on our Cannabis Use variable - of those, 13 failed to meet quality control (QC) standards in FreeSurfer-based image-analysis pipeline. Our final data set for our CU variable consisted of 471 participants. For our analyses of Cumulative Dose, data regarding cannabis exposure were only available for 105 of 508 participants (105 of 126) Early Cannabis Users, 3 of whom had unusable brain data due to QC failure. Our final data set for our CD variable consisted of 102 participants. See Table 4 for a summary of participant exclusions.

Table 4. Final data set.

Excluded Participants			
		Cannabis Use	**Cumulative Dose**
Inconsistent Data			
Reported "Yes" 1 time		6	n/a
Reported "Yes" 2 times		8	n/a
Reported "Yes" to ever tried but "Never" to last time use		1	n/a
	Total	15	–
Missing Data			
All previous reports are "No" & missing last T7		2	n/a
All previous reports are "No" & Missing (at least) T5 & T6		7	n/a
	Total	9	–
	Total	24	–
MRI Quality Control			
		Cannabis Use	**Cumulative Dose**
Failure		13	3
	Total	13	3
Final Data Set			
		Cannabis Use	**Cumulative Dose**
Scanned Participants		508	105
Excluded Participants		24	0
MRI Quality Control Failure		13	3
	Total	471	102

Table 5. Comparison groups for independent variables.

	Cannabis Use				Cannabis Dose[2]			
	Never	Ever[1]			Lowest	Low	High	Highest
		Late[3]	Early[4]		*<5*	*6 to 20*	*20 to 60*	*>60*
n	224	122	125	*n*	41	25	20	16

[1] Participants who reporting using cannabis at least once by 18 to 21 years of age.

[2] Total number of "times" participants had smoked smoked cannabis by 16.5 years.

[3] Participants who reported first using cannbis after the age of 16.5.

[4] Participants who reported first using cannbis by the age of 16.5.

2.2.3 Dependent measures

Magnetic resonance imaging (MRI). MRI scans were acquired with a General Electric 3T magnet, using an 8-channel, receiver-only head coil. 3D fast spoiled gradient-echo (FSPGR) sequence enabled the collection of T1-weighted MR images following these parameters: oblique axial orientation (plane passing through the anterior-posterior commissures), 1-mm isotropic, field of view 256x192x210mm, TR=7.9 ms, TE=3.0 ms, TI=450ms and Flip angle=20deg.

Cortical thickness. Group differences were examined in cortical thickness, our main dependent variable of interest. This measure was generated using Freesurfer 5.3.0 (Charlestown, MA, USA), which segments and maps the entire cerebral cortex, white matter and 35 subcortical structures and then assembles meshes of the brain using approximately 160,000 triangles containing geometric and topological information of the pial surface, gray matter, and white matter independently, for every MR image **(**Fischl, 2012). Local cortical thickness represents the distance between the position of homologous vertices in the pial and gray/white surfaces. A correspondence between the cortical surfaces across participants is established using a nonlinear alignment of the principal sulci in each participant's brain with an average brain (Desikan et al., 2006). Regarding its relative sensitivity as a parameter of brain morphology, cortical thickness is considered to be more sensitive than voxel-based morphometry due to a higher signal-to-noise ratio (Hutton, Draganski, Ashburner, & Weiskopf, 2009).

***CB1* gene expression-weighted cortical thickness.** In the event group differences in brain structure exist, there are several plausible mechanisms that may mediate the relationship between cannabis use and brain structure; these will be explored in the discussion section. One of such mechanisms is a direct pharmacological effect of the active component of cannabis, namely, 9(delta) tetrahydrocannabinoid (THC), which is mediated by cannabinoid receptor 1 (*CB1*). For this reason, we investigated the influence of *CB1* receptor density on the relationship between cannabis use and cortical thickness. To accomplish this, we used gene expression data as a proxy measure of *CB1* receptor density throughout the human cortex. Using gene expression data, we compared group differences in cortical thickness based on *CB1* density in two different ways. First, we informed our thickness data by gene expression data to create a *CB*- weighted average thickness to generate a whole brain measure of cortical thickness that was weighted according to regional variations in *CB1* receptor density. Second, we compared group differences in cortical thickness between terciles of cortical regions with high, moderate, and low densities of *CB1* receptors. The gene expression data and the process by which we derived our *CB1* related dependent measures of cortical thickness are detailed in the following sections.

2.2.4 Deriving dependent measures

Allen Brain Atlas. Our resource for *CB1* receptor gene expression data was the online databank of the Allen Institute of Brain Science (Shen, Overy, & Jones, 2012). The Allen Human Brain Atlas is a microarray-based assay of the expression of all genes in a number of regions in the brains of six donors who ranged in age, ethnicity and handedness (Table 6). Samples were extracted from the left hemisphere from all donors, while only 2 of these 6 donors yielded samples from both hemispheres. T1-weighted whole brain scans were acquired *post mortem* and segmented using FreeSurfer's pipeline prior to being carefully anatomically labeled and dissected for genome-wide gene expression profiling. Two methods were used to dissect samples: (1) a scalpel-based manual macrodissection method primarily for cortical and other relatively large uniform samples; and (2) laser microdissection (LMD) for small or oddly-shaped structures such as subcortical or brainstem areas. Approximately 500 anatomically discrete samples per hemisphere were collected from cerebral cortex, subcortex, cerebellum and brainstem of each brain of each donor and profiled for genome-wide gene expression using a custom Agilent 8x60K cDNA array chip. A combined total of approximately 4,000 unique anatomic samples were assayed using 60,000 gene-expression probes per sample. The project spanned approximately three years to process and collect array data on all samples, with data being made available to the public throughout the project.

Table 6. Allen Brain Atlas donor characteristics

Donor	Age	Sex	Ethnicity	Handedness	Hemispheres Sampled
1	57	Male	Caucasian	Cross-dominant	Left
2	49	Female	Hispanic	Right	Left
3	31	Male	Caucasian	Right	Left
4	55	Male	Caucasian	Right	Left
5	24	Male	African American	Left	Left & Right
6	39	Male	African American	Left	Left & Right

Microarray gene expression data, based on 89 probes for *CB1* expression, were quantified and normalized across donor brains. The Allen Brain Atlas has combined Nissl staining, anatomical labeling, dissection, and MRI techniques to the end of uniting gene expression data of a given anatomical sample with MRI data via mapping it to 3D space. We downloaded these data from the online Allen data portal at http://human.brain-map.org/. Our gene expression data consisted of a list of samples, each paired with information regarding the sampled hemisphere, the sampled anatomical region, the sampled donor, a measure of gene expression, and a corresponding set of MNI coordinates (Mazziotta et al., 2001). MNI coordinates represent the approximate center of the sample. Importantly, MRI data in both our study and the Human Brain Atlas study were similarly processed using FreeSurfer software, and located in the same MNI standardized stereotaxic space. Therefore, gene expression data and cortical thickness shared common 3D space; we exploited this shared link to connect both datasets.

Mapping Allen Brain Atlas to FreeSurfer. Gene expression data from the Allen Brain Institute (ABI) database include corresponding sets of MNI coordinates for every gene expression sample, representing the approximate center of the sample. FreeSurfer was used to process MRI data, parcellate, and label 68 different regions of the cortex in MNI space for every ALSPAC participant. Similarly, the ABI used FreeSurfer to process MRI of the post-mortem brains, and map the 3D location of gene expression samples for each donor - importantly, also in MNI space. Thus, MNI space served as the common link connecting our cortical thickness data with the gene expression data.

In order to inform cortical thickness by gene expression data from the Allen Institute database, we required a corresponding FreeSurfer label for each of these sets of coordinates (gene expression sample location). To accomplish this, we ran FreeSurfer (version 5.3) pipeline on the ICBM152 template (identical to the "MNI space"). By doing so, we generated a three-dimensional map of a standardized brain in MNI space, which had been processed through FreeSurfer's pipeline parcellating the brain into

anatomically labeled regions. The result was a map of an every voxel in MNI space, which was either assigned no label, or a FreeSurfer label. To read this map, a computer algorithm was written and executed to retrieve the assigned FreeSurfer label for every ABI sample location's MNI coordinates in the parcellated and labeled MNI template brain. We encountered two issues. First, a number of ABI samples fell outside of the boundaries of regions as parcellated by FreeSurfer pipeline and were not assigned a FreeSurfer label by our algorithm. Second, of those assigned a FreeSurfer label, there were a number of discrepancies between our generated FreeSurfer label, and the anatomical label of the gene sample assigned and provided for each gene sample location by the ABI. These errors were not unanticipated; subtle discrepancies were expected due to ABI providing MNI coordinates in donors' native space, resulting in an imperfect overlap with our ICBM152 template. This process therefore served as a step providing with a very close approximation of the FreeSurfer label in which a gene sample coordinate would fall within the boundaries of. We created and adhered to a set of quality control standards to resolve both issues, detailed below.

Quality control. As discussed (see Mapping Allen Brain Atlas to FreeSurfer), there were a number of gene samples that were unclassified, or misclassified in terms of FreeSurfer labels. We attempted to recover unclassified samples and correct misclassified samples due to error, recognizing our use of icbm152 template brain yielded a close approximation but imperfect overlap. In doing so, we operated under the assumption that the ABI anatomical label was accurate given extensive efforts by the ABI to provide detailed, precise and accurate anatomical labels for gene samples (details provided at www.brain-map.org).

Unclassified samples. Gene samples were unassigned if they fell outside the boundaries of FreeSurfer regions, so our algorithm was adjusted to output the label of the nearest FreeSurfer region to a set of coordinates, as well as the number of voxels away it was from that region. We moved forward with all samples falling up to 3 voxels away from a FreeSurfer region. Our threshold of 3 voxels was based jointly on minimizing the distance a sample to the nearest FreeSurfer region to be conservative and maximizing the number of samples we would gain per donor and per hemisphere for each cortical region. With this threshold, we recovered 482 voxels. With these samples, 100% of right hemisphere region data were based on the maximum number of donors possible (n = 2), and 94% of left hemisphere region data were based on the maximum number of donors possible (n = 6) - the 3 regions with only 4 of 6 donors contributing were the 3 smallest FreeSurfer cortical parcellations and therefore it would be impossible for these regions to have been sampled as many times as much larger regions. We are therefore confident each of our regions had sufficient samples to be representative of the ABA sample at the end of our process. The minimum number of samples contributing to any region was 8. The mean number of samples contributing to a given region was 44. In total, our final list was comprised of 1525 samples. At

the end of this process, we were left with a list of all samples falling in or within 3 voxels away from a FreeSurfer region - each with both ABI-assigned anatomical labels, and FreeSurfer labels.

Misclassified samples. Anatomical labels for each sample were visually reviewed and compared for correspondence. Two types of discrepancies between samples were identified: cortical/subcortical inconsistencies and within-cortex inconsistencies. Cortical/subcortical inconsistencies were discrepancies in assignment of a sample as either cortical or subcortical - for example if one of the labels for the sample was hippocampal and thus subcortical, while the other was parahippocampal, and thus cortical. Such inconsistencies were resolved by defaulting to the Allen anatomical label as correct; if the ABI label determined the sample was subcortical, we relabeled it as such. Within-cortex inconsistencies occurred due to idiosyncrasies between nomenclature. FreeSurfer has its own set of labels, for example "the label bankssts" does not have a direct equivalent in ABI labels. Additionally, ABI created their own anatomical labeling system based on a compilation of various expert sources, and was often more fine-grained, for example "Heschl's gyrus" does not have an exact equivalent in FreeSurfer labels. To determine the most appropriate FreeSurfer cortical label and when necessary replace a label, the Automatic Anatomical Labeling (AAL; ref) template (importantly, also in MNI space) was overlayed on our icbm152 template brain and used a reference. Recently, Goel and colleagues used AAL as a reference, and starting point in their creation of bridge between FreeSurfer's and the ABI's anatomical distinctions, to the same end of mapping ABI coordinates to FreeSurfer parcellations (Goel, Kuceyeski, LoCastro, & Raj, 2014). Our final list included 1525 samples with a cortical FreeSurfer label, and a gene expression value.

Mean CB1 weighted average cortical thickness. We chose to use a method of weighting as a way of informing our whole-brain cortical thickness measure by *CB1* expression. To create our CB1-Weighted thickness measure, we calculated a weighted average of cortical thickness using the same thickness data with which we used to calculate the mean (unweighted) cortical thickness. Thus, the resulting weighted thickness reflects the contribution of each cortical region that varies in proportion to their on their relative *CB1* expression. Based on our list of samples, we calculated the median of *CB1* expression values for each of the 68 (34 left hemisphere, 34 right hemisphere) cortical regions. We used the median instead of the mean to protect against extreme values. Weights were assigned based on the median value of *CB1* expression for each cortical region; specifically, the quotient of a region's median *CB1* expression by the total sum of its median expression for all cortical regions served as our *CB1* expression weights. In calculating our weighted thickness, we treated each of the 68 cortical regions independently so the *CB1* weight of a given region in one hemisphere was independent of that in the other hemisphere. In following the formula for a weighted average, thickness measures for each cortical region were multiplied by the corresponding *CB1* weight for that region, and finally summed to produce our *CB1*-weighted bilateral average. This was calculated for all participants.

***Regional variation in CB1 density*.** We compared the effect of CU on cortical thickness and the effect of CD on cortical thickness across cortical regions exhibiting either low, moderate, or high *CB1* receptor density, as indexed by gene expression. In the way that a weighted average thickness allowed us to evaluate the influence of *CB1* receptor density across the whole brain, this measure allowed us to compare the influence of *CB1* receptor density between sets of regions with varying *CB1* density.

Since a maximum of only 2 donors contributed to right hemisphere samples in the ABI, as compared with a maximum of 6 donors for left hemisphere samples (see Table 5.), we only used gene expression data for left-hemisphere regions as a more conservative approach. Within the 34 FreeSurfer regions in the left hemisphere, 5 cortical regions had less than all 6 donors contributing samples. We excluded one region as it had only 2 donors (bankssts). The remaining 33 regions were included in our analysis; of those, 29 regions had samples from all 6 donors, while 2 had samples from 5 donors, and 2 had samples from 4 donors. These 33 regions were ranked according to *CB1* gene expression then divided into terciles. The lowest, middle, and highest terciles served as our "Low", "Moderate", and "High" *CB1* density groups, respectively. Cortical regions comprising each of the terciles with their corresponding *CB1* gene expression values can be found in Table 7 below.

Table 7. Cortical regions and gene expression in High and Low *CB1* density groups.

Low CB-1		Moderate CB-1		High CB-1	
cuneus	4.77	lateraloccipital	5.94	superiortemporal	6.36
lingual	4.89	bankssts	5.95	entorhinal	6.41
inferiorparietal	5.08	paracentral	5.99	superiorfrontal	6.51
parstriangularis	5.22	supramarginal	6.12	parsopercularis	6.62
pericalcarine	5.38	parahippocampal	6.12	lateralorbitofrontal	6.63
transversetemporal	5.42	insula	6.23	isthmuscingulate	6.64
superiorparietal	5.66	fusiform	6.23	medialorbitofrontal	6.67
inferiortemporal	5.66	caudalmiddlefrontal	6.23	temporalpole	6.72
precuneus	5.73	precentral	6.24	rostralmiddlefrontal	6.82
parsorbitalis	5.79	middletemporal	6.24	caudalanteriorcingulate	6.85
postcentral	5.81	posteriorcingulate	6.27	rostralanteriorcingulate	6.99

2.3 Hypotheses

Three separate sets of analyses were conducted, each comparing group differences in cortical thickness based on our two cannabis-related independent variables: cannabis use and cumulative dose. First we compared cortical thickness values, then *CB1* weighted average cortical thickness, and finally cortical thickness values in regions with low, moderate, and high *CB1* density.

Our hypotheses were informed by the literature reviewed above, especially by those most analogous to our own in terms of study design. In comparing early onset, late onset, and non cannabis users, we hypothesized both early users and late users would have lower mean cortical thickness relative to non users; this was based on the majority of studies investigating grey matter differences reporting reduced grey matter in cannabis users as compared with non users reporting, (Batalla et al 2013; Lorenzetti et al., 2010; Martin-Santos et al., 2010; Rochetti et al, 2013). Our hypotheses were additionally founded on studies by Mata et al.(2010) and Lopez-Larson et al.(2011), which reported group differences using the same brain metric of cortical thickness; we hypothesized our results would be at least partly in line with these studies, both of which reported thinner cortical regions in cannabis users.

Moreover, we further hypothesized the difference in thickness would be magnified in early onset users, with early onset users (before 16.5 years) exhibiting a thinner cortex as compared with late onset users, having the thinnest cortex of the 3 comparison groups. This hypothesis was founded on several studies finding age of cannabis use onset to differentiate, as well as correlate with structural outcome measures in grey and white matter (Arnone et al., 2008; Batalla et al., 2014; Wilson et al., 2000).

In our analyses of cumulative dose in early users, we hypothesized to find an association with cortical thickness, where groups with higher cumulative dose would exhibit a thinner cortex. We further hypothesized this association would be stronger than that of our cannabis use measure, as we expect cumulative dose to be a more robust and representative measure of cannabis. In allowing for the comparison of heavier versus slighter users, we expect to be more likely to detect differences, as well as be more consistent with previous literature, which often compared non users to heavy or chronic users of cannabis.

Finally, we expect the associations between cannabis use and dose with thickness will be stronger in regions with higher CB1 density as compared with moderate and lower densities. If the direct pharmacological effects of cannabis were driving the association between cannabis and grey matter changes, we would expect our mean *CB1* weighted average to perform better in our GLM model, explaining more variance and having a greater level of significance as compared with our mean whole brain CT measure. Our hypothesis is guided by a body of evidence supporting the role of pharmacological effects. For example, binding of THC to CB1 receptors are necessary to induce cannabis' subjective

effects, and there is an apparent concordance between CB1 receptor localization and the subjective effects of cannabis.

It should be noted that while our hypotheses were directed by previous studies, we recognize that findings in the existing literature are inconsistent and far from conclusive, thus the overarching motivation behind this study was to determine whether or not there group differences in structural brain properties existed based on cannabis exposure. This is especially true regarding our analyses of CB1 receptor density. Previous studies have noted post hoc that brain regions differing in cannabis users tend to be CB1 rich, or have alternatively chosen CB1 rich ROIs a priori - both of which are bias in their interpretation and assumption respectively, of CB1 receptors as a cause or mediator of structural differences associated with cannabis use (Bigeon & Kerman, 2001; Katona et al., 2001; Mato et al., 2003; Rais et al., 2010). While there is evidence to suggest pharmacological effects of cannabis are in part responsible for the apparent relationship between cannabis use and grey matter differences, we primarily conducted analyses from an exploratory perspective.

2.4 Statistical Model

Our hypotheses that cortical thickness would differ significantly between our CU groups, as well as our CD groups were tested using simple analysis of variance. To explore further the relationship between cortical thickness and our cannabis use variables, we designed a general linear model (GLM) univariate analysis of covariance in an effort to test the stability of significant relationships when controlling for potential confounders. Our intended goal was to observe changes in the predictive value, and explained variance in our models, and second to test whether or not the relationship between cortical thickness and remained constant after adjusting for these factors and thus provide evidence that observed relationships were not likely attributable to potential confounders.

Included in our model were variables we considered to be known or potential confounders based on a review of the literature (see previous section). Confounders were separated into five conceptual categories: biological, environmental, behavioural, early substance use, and current substance use. We conducted multiple ANCOVAs, adding one category of confounders to our original ANOVAs, and each subsequent ANCOVA. Categories were conceptually based on the chronological order in which they could possibly influence cortical thickness. See Table 8. below for details regarding our ANCOVA models.

Table 8. Category of confounders added to each analysis of covariance model

Model	Category	New Confounder(s)	Measure
1	**Biological**	Age	In months.
2	**Environmental**	Maternal social class	Reported by mother at 32 weeks gestation.
3	**Behavioural**	Conduct disorder symptoms	Likelihood of being diagnosed with Conduct Disorder (CD) based on symptoms present at 10 years old. Likelihood calculated as a percent by a computerized program adhering to ICD and DSM IV diagnostic criteria. Symptoms were reported by parent.
4	**Early Substance Use**	Cumulative cigarettes smoked Cumulative binge drinking	At 15.5 years old.
5	**Current Substance Use**	Cumulative cigarettes smoked, Frequency of binge drinking, Frequency of cannabis use	At the time of scan, when participants were between the ages of 18 to 21.

Chapter 3
Results

3 Results

We performed several sets of statistical analyses to test our hypothesis that exposure to cannabis during adolescence would have an effect on brain structure in young adulthood. Statistical analyses were conducted on the final data set of participants whose self-report and MRI data both met the quality control standards detailed in the previous chapter, Chapter Two: Methodology. Prior to all analyses, any statistical outliers falling outside 3 standard deviations from the mean were removed. All statistical analyses were conducted using IBM's SPSS Statistics version 2.

3.1 Sample Characteristics

Cannabis use. At the time of scanning, CU groups differed in age by less than 5 months, with Never Users being the youngest (M = 233.7, SD = 10), Late Users as the oldest (M = 238.3, SD = 10), and Early Users falling in between (M = 235.1, SD = 10), $F(2, 468) = 8.23$, $p = <.01$. Cannabis User groups did not differ in level of Maternal Social Class at 32 weeks gestation ($\chi 2 = 4.0$, $p = .86$), or conduct disorder symptoms at 10 yr of age ($\chi 2 = 9.9$, $p = .27$). Regarding early substance use (at 15.5 yr of age), groups differed in their total number of cigarettes smoked ($\chi 2 = 151.5$, $p = <.01$), and their binge drinking ($\chi^2 = 90.2$, $p = <.01$). Participants similarly differed in substance use at the time of scan in terms of total lifetime cigarettes smoked ($\chi 2 = 280.5$, p = <.01), binge drinking at scan ($\chi 2 = 56.1$, $p = <.01$), and frequency of cannabis use at scan ($\chi 2 = 300.1$, p = <.01). As shown in Table 9., all substance use-related variables, our Early Users and Late Users groups more frequently endorsed using substances more frequently or more in total.

Cannabis dose. The four CD groups differed in less ways than CU groups, only differing on variables regarding smoking behaviour. CD groups did not differ in age at scan ($F(3, 98) = .2$, $p = .9$), level of Maternal Social Class at 32 weeks gestation ($\chi^2 = 17.9$, $p = .12$), likelihood of conduct disorder ($\chi^2 = 6.6$, $p = .7$), frequency of binge drinking at 15.5 years ($\chi^2 = 28.9$, $p = <.12$), or frequency of binge drinking at 18 to 21 ($\chi^2 = 15.5$, $p = <.42$). Participants did significantly differ in terms of their total number of cigarettes smoked at 15.5 ($\chi^2 = 25.2$, $p = <.05$) and 18 to 21 ($\chi^2 = 29.8$, p = .01), as well as their frequency of cannabis smoking at 18 to 21 ($\chi^2 = 36.6$, p = <.01). As shown in Table 10., similar to the CU group differences, participant exhibiting greater use of cannabis also tended to use other substances (cigarettes and alcohol) to a greater extent.

Table 9. Differences in confounder variables across cannabis use comparison groups

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Covariate	Group					
	Never	Late[1]	Early[2]			
Age at Scan[3]	M(SD)	M(SD)	M(SD)	**n**	**F**	***p***
	233.8(10)	238.3(10)	235.1 (10.2)	**471**	**8.2**	**<.01**
Maternal Social Class[4]				**n**	**χ^2**	**p value**
I	11.7%	7.5%	14.3%	**429**	**6.7**	**0.75**
II	41.6%	38.3%	41.0%			
III (non-manual)	35.5%	40.2%	38.1%			
III (manual)	5.6%	6.5%	2.9%			
IV	4.1%	5.6%	3.8%			
V	1.5%	1.9%	0.0%			
Conduct Disorder predicted at age 10[5]				**n**	**χ^2**	***p***
~0.5%	77.3%	72.0%	64.5%	**470**	**10.1**	**0.26**
~3%	20.9%	27.1%	33.1%			
~15%	.9%	.8%	.8%			
~50%	.5%	0.0%	0.0%			
>70%	0.50%	0.0%	1.70%			
Cumulative Binge Drinking Instances at age 15.5[6]				**n**	**χ^2**	***p***
0	72.0%	56.0%	28.9%	**448**	**90.2**	**<.01**
1-2	15.2%	18.1%	19.0%			
3-5	5.2%	6.9%	9.1%			
6-9	3.8%	8.6%	12.4%			
10 - 20	1.9%	6.9%	9.9%			
20-39	1.4%	3.4%	9.9%			
40-99	0.5%	0.0%	9.1%			
100+	0.0%	0.0%	1.7%			
Cumulative Cigarettes Smoked at age 15.5				**n**	**χ^2**	***p***
0	96.2%	89.7%	47.5%	**450**	**151.2**	**<.01**
<5	2.8%	7.7%	9.8%			
5 - 19	0.5%	1.7%	10.7%			
20-49	0.0%	0.9%	10.7%			
50-99	0.5%	0.0%	9.0%			
100+	0.0%	0.0%	12.3%			
Frequency of binge drinking at age 18 to 21[7]				**n**	**χ^2**	***p***
Never	17.4%	1.6%	4.8%	**471**	**56.1**	**<.01**
Once or twice	17.0%	12.3%	6.4%			
Less than monthly	20.5%	14.8%	14.4%			
Monthly	25.4%	32.8%	28.8%			
Weekly	19.2%	38.5%	44.8%			
Daily or almost daily	.4%	0.0%	.8%			
Frequency of Cannabis Use at 18 to 21 years[8]				**n**	**χ^2**	***p***
Never	100.0%	34.4%	20.8%	**471**	**300.1**	**<.01**
Monthly or less	.0%	54.9%	43.2%			
2 to 4 times per month	.0%	5.7%	19.2%			
2 to 3 times per week	0.0%	3.3%	8.0%			
4+ times per week	.0%	1.6%	8.8%			

[1]Early Users smoked cannabise by the age of 16.5.

[2]Late Users smoked cannabise after the age of 16.5.

[3]Age presented in months.

[4]Percentage in ascending categories.

[5]Percent likelihood child would be diagnosed as having conduct disorder by ICD-10, DSM standards; parent-informed; computer predicted.

[6]Number of times adolescent drank 5 or more times in

[7]Number of times adolescent drank 6 or more times in

[8]Frequency within the last year.

Table 10. Differences in confounder variables across cannabis dose comparison groups.

Covariate	Group						
	Lowest	Low	High	Highest			
Age at Scan[3]	M(SD)	M(SD)	M(SD)	M(SD)	**n**	**F**	***p***
	233.6 (8.8)	234.6 (11.6)	234.2 (11.2)	235.8 (9.6)	101	0.2	0.91
Maternal Social Class[4]					**n**	**χ^2**	***p***
I	14.7%	5.0%	40.0%	7.1%	83	17.9	0.12
II	41.2%	55.0%	20.0%	57.1%			
III (non-manual)	38.2%	35.0%	26.7%	28.6%			
III (manual)	2.9%	0.0%	13.3%	0.0%			
IV	2.9%	5.0%	0.0%	7.1%			
V	0.0%	0.0%	0.0%	0.0%			
Conduct Disorder predicted at age 10[5]					**n**	**χ^2**	***p***
<0.1%	0.0%	0.0%	0.0%	0.0%	96	6.6	0.70
~0.5%	70.0%	58.3%	72.2%	71.4%			
~3%	30.0%	33.3%	27.8%	28.6%			
~15%	0.0%	4.2%	0.0%	0.0%			
~50%	0.0%	0.0%	0.0%	0.0%			
>70%	0.50%	0.0%	1.70%	0.0%			
Cumulative Binge Drinking Instances at age 15.5[6]					**n**	**χ^2**	***p***
0	35.0%	20.8%	20.0%	26.7%	99	28.9	0.12
1-2	27.5%	25.0%	20.0%	0.0%			
3-5	10.0%	12.5%	5.0%	0.0%			
6-9	10.0%	12.5%	15.0%	13.3%			
10 - 20	7.5%	12.5%	20.0%	6.7%			
20-39	2.5%	4.2%	5.0%	33.3%			
40-99	5.0%	12.5%	10.0%	20.0%			
100+	2.5%	0.0%	5.0%	0.0%			
Cumulative Cigarettes Smoked at age 15.5					**n**	**χ^2**	***p***
0	65.0%	60.0%	40.0%	20.0%	**100**	**25.2**	**<.05**
<5	7.5%	8.0%	5.0%	20.0%			
5 - 19	7.5%	8.0%	10.0%	13.3%			
20-49	10.0%	16.0%	10.0%	0.0%			
50-99	0.0%	4.0%	20.0%	26.7%			
100+	10.0%	4.0%	15.0%	20.0%			
Lifetime Cumulative Cigarettes Smoked at Scan					**n**	**χ^2**	***p***
0	19.5%	0.0%	10.0%	0.0%	**102**	**29.8**	**0.01**
Less than 5	14.6%	4.0%	0.0%	0.0%			
5 - 19	12.2%	4.0%	5.0%	0.0%			
20-49	7.3%	16.0%	5.0%	0.0%			
50-99	2.4%	8.0%	0.0%	12.5%			
100+	43.9%	68.0%	80.0%	87.5%			
Frequency of binge drinking at age 18 to 21[7]					**n**	**χ^2**	***p***
Never	7.3%	0.0%	10.0%	6.3%	102	15.5	0.42
Once or twice	7.3%	8.0%	5.0%	6.3%			
Less than monthly	24.4%	8.0%	0.0%	12.5%			
Monthly	24.4%	32.0%	30.0%	18.8%			
Weekly	36.6%	52.0%	50.0%	56.3%			
Daily or almost daily	.0%	0.0%	5.0%	.0%			
Frequency of Cannabis Use at 18 to 21 years[8]					**n**	**χ^2**	***p***
Never	39.0%	8.0%	5.0%	6.3%	**102**	**36.6**	**<.01**
Monthly or less	46.3%	64.0%	35.0%	31.3%			
2 to 4 times per month	7.3%	16.0%	40.0%	25.0%			
2 to 3 times per week	4.9%	12.0%	10.0%	12.5%			
4+ times per week	2.4%	0.0%	10.0%	25.0%			

[1]Early Users smoked cannabise by the age of 16.5.

[2]Late Users smoked cannabise after the age of 16.5.

[3]Age presented in months.

[4]Percentage in ascending categories.

[5]Percent likelihood child would be diagnosed as having conduct disorder by ICD-10, DSM standards; parent-informed; computer predicted.

[6]Number of times adolescent drank 5 or more times in

[7]Number of times adolescent drank 6 or more times in

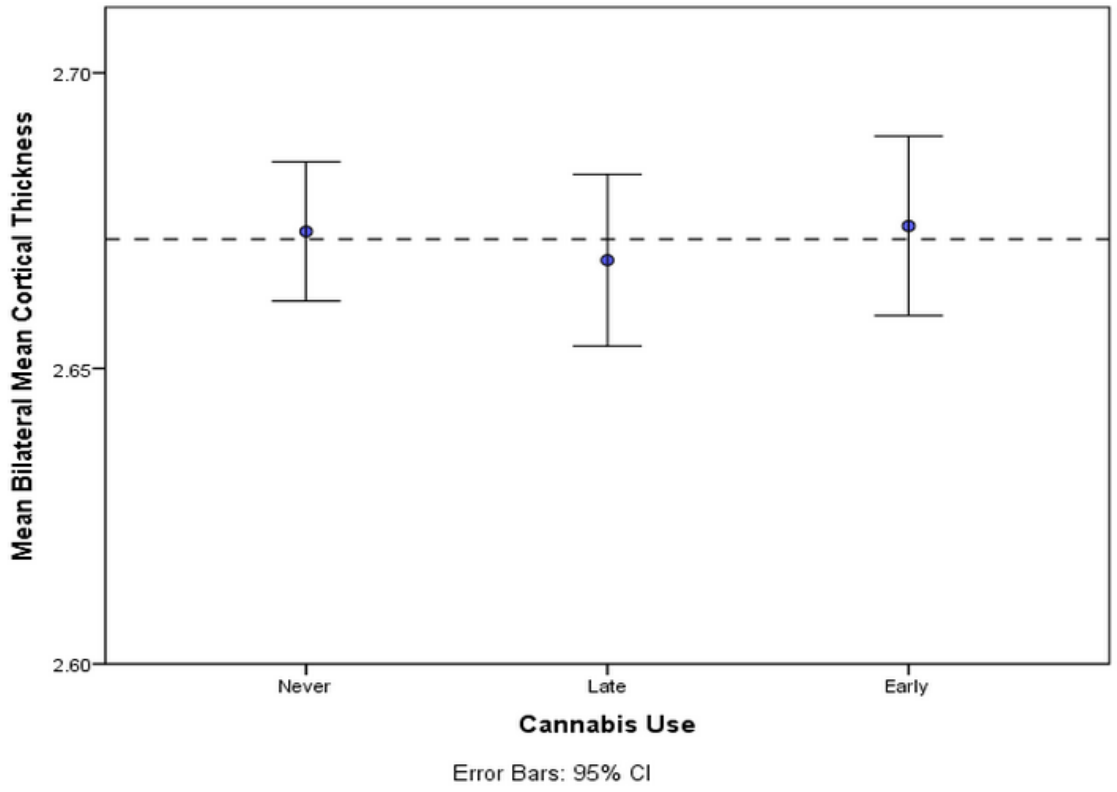
[8]Frequency within the last year.

3.2 Mean Cortical Thickness

Analysis of variance. After removal of one statistical outlier, mean cortical thickness was equal to 2.67 mm in the final data set (n=470), which was consistent with the mean of 2.68 for the 101 participants in the smaller subset of participants for whom cannabis dose was analyzed (M = 2.67, SD = .08; M = 2.68, SD = .08). Cortical thickness was normally distributed in the full sample and the subset for whom cannabis dose was analyzed. Homogeneity of variance was confirmed by insignificant Levene's tests across cannabis use groups (2, 467), W = .64, p = .53 and across cannabis dose groups (3, 97), W = 2.4 , p = .07 alike. Upon confirmation our data conformed to necessary assumptions, we performed two ANOVAs testing for effects of cannabis use and cannabis dose.

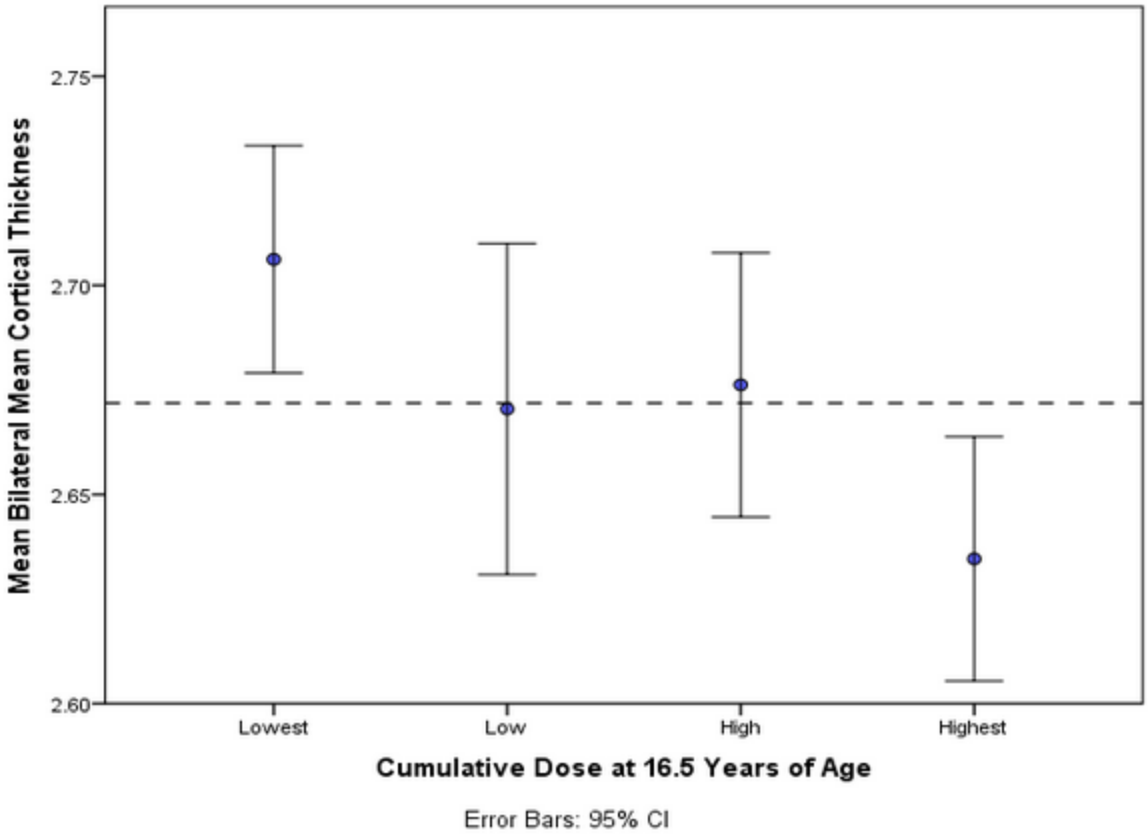
Cannabis use. We tested if mean cortical thickness differed by our cannabis use factor in our sample (n = 470) using analysis of variance (ANOVA), finding no statistical differences between adolescents who had never smoked cannabis, those with early onset of cannabis use, and those with late onset cannabis use $F(2, 467) = .17$, p = .85. This held true when corrected for age $F(2, 466) = .04$, p = .97. We fail to reject the null hypothesis of equal means across our different cannabis use participant groups.

Figure 1. Mean bilateral mean cortical thickness (age corrected) across cannabis use groups.



Cannabis dose. An ANOVA was then run testing an effect of Cannabis Dose in our sample subset (n = 101), finding significant group differences between the different factor levels F (3, 97) = 3.1, p = .03. This held true when corrected for age F (3, 96) = 3.0, p = .04. Post-hoc power analysis confirmed our significance test of the effect of CD at the α = .05 level was powerful, having power of .7. Results indicate a moderate effect of CD; 9% of total variance in mean cortical thickness (η^2 = .09) can be attributed to CD.

Figure 2. Mean cortical thickness (age corrected) across cannabis dose groups.



Post-hoc pairwise comparisons using the conservative Bonferroni test showed the mean thickness of the group with the highest cumulative cannabis dose (M = 2.63, SD = .05) was significantly lower than that of the group with the lowest cumulative cannabis dose (M = 2.71, SD = .09) at the .05 level, correcting for FWE, p = .03. Given the significant difference exhibited by the lowest and highest CD groups, a calculation of Cohen's (d = 1) using the software program G*Power revealed a large effect size (Erdfelder, Faul, & Buchner, 1996); the means of the lowest and highest CD groups differed by 1 standard deviation. Given this, we have strong evidence suggesting we reject the null hypothesis of equal thickness means across our Cannabis Dose participant groups.

General Linear Model. Two identical sets of general linear model (GLM) analyses of covariance (ANCOVA) were run, one for each of our independent variables: Cannabis Use (CU) and cannabis Cumulative Dose (CD).

Cannabis Use. In our models predicting mean cortical thickness, our CU predictor variable only just reaches significance level in the final model including all possible predictor variables, F (2, 314) = 3.0, p = .05. Partial eta-squared showed our CU variable accounted for 2% of variance in mean cortical thickness, η_p^2 = .02.

Cannabis Dose. In our analyses of mean cortical thickness, the significant association observed between CD and CD from our ANOVA was maintained throughout all our ANCOVA univariate GLM models, with the exception of Model 4 (addition of early substance use), F (3, 54) = 2.3 , p=.09. Significance of CD was recovered in Model 5, after adjusting for current substance use variables, F (3, 41) = 3.0, p=.04. CD reached a peak significance in Model 2, in which we controlled for age at scan and environmental factors (social class), F(3, 73) = 3.8, p = .015. Using CD as a predictor, our final model with all variables accounted for the most variance in mean cortical thickness, at 62% (R^2 = .62); CD accounted for 18% of variance in this model according to partial eta-squared, η_p^2 = .02, a contribution surpassed only by lifetime smokes by the age of 16.5, and current binge drinking in our final model.

3.3 CB1 Weighed Cortical Thickness

Analysis of variance. Mean *CB1* weighted cortical thickness was equal to 2.78 for all 470 participants in the final data set (M = 2.78, SD = .09). For the subset of 101 participants for whom cannabis dose was analyzed, mean *CB1* weighted thickness was equal to 2.79 (M = 2.79, SD = .09).

Cannabis Use. Similar to predicting mean CT, no statistical differences between adolescents who had never smoked cannabis, those with early onset of cannabis use, and those with late onset cannabis use $F(2, 467) = .14$, $p = .87$. We fail to reject the null hypothesis of equal means across our different Cannabis Use participant groups. Results are detailed in Table 11 below.

Cannabis Dose. In our analyses of *CB1* weighted CT, we can see the effect of CD is approximately equal (see above) to our original, unweighted CT values, F (3, 97) = 3.17 p = .028. Equal coefficients of determination for our *CB1* weighted CT and CT show the same amount of variance was accounted for (η^2 = .09). Similar to our mean CT, post-hoc pairwise comparisons once again showed a significant difference between the lowest and highest dose groups according to a Bonferroni test , though very slightly more significant, p = .02. Results are detailed in Table 12. below.

General Linear Model. In repeating our 5 models again for our *CB1* weighted average CT, we see no significant association for our CU predictor variable.

Cannabis Use. Relative to mean cortical thickness, our models were for the most part similar in that they performed poorly in predicting *CB1* weighted cortical thickness, but differed in that cannabis use did not reach significance in predicting *CB1* weighted CT in the final model $F(2, 314) = 1.9$ $p = .15$. Results of our ANCOVA models for *CB1* weighted CT are shown below in Table 11.

Cannabis Dose. Cannabis dose demonstrated significant contributions in predicting *CB1* weighted CT for models that controlled for age at scan, environment, and behaviour, but significance did not survive the addition of other substance use variables in model 4 and 5. The performance of our models for *CB1* weighted CT and mean CT are roughly equivalent in terms of variance accounted for by our predictors and models but overall more variance can be accounted for in mean CT values than can be for our generated *CB1* weighted CT variable. Results of our ANCOVA models for both bilateral mean CT, and *CB1* weighted average CT are shown below in Table 12.

Table 11. Results of cannabis use ANCOVA models predicting cortical thickness.

			Cortical Thickness			
			Bilateral Mean		CB-1 Weighted	
			M = 2.67 (.08)		M = 2.78 (.09)	
		n	p	R^2	p	R^2
Model 1: Cannabis Use[1]	Cannabis Use	470	0.85	0.00	0.87	0.00
Model 2: + Age [2]	Cannabis Use	470	0.96	0.02	0.93	0.02
	Age		< .01[a]		0.03[a]	
Model 2: + Environment	Cannabis Use	391	0.81	0.03	0.82	0.02
	Age		0.01[a]		0.08	
	Maternal Social Class		0.26		0.25	
Model 3: + Behaviour	Cannabis Use	368	0.80	0.03	0.81	0.02
	Age		0.02[a]		0.09	
	Maternal Social Class[3]		0.48		0.53	
	Conduct Disorder Symptoms (10 yrs)		0.95		1.00	
Model 4: + Early Substance Use	Cannabis Use	351	0.05	0.10	0.09	0.09
	Age		0.02[a]		0.10	
	Maternal Social Class		0.17		0.30	
	Conduct Disorder Symptoms (10 yrs)[4]		0.66		0.83	
	Cumulative Cigarettes Smoked (15.5 yrs)		0.03[a]		0.05[a]	
	Cumulative Binge Drinking (15.5 yrs)[5]		0.28		0.15	
Model 5: + Current Substance Use	Cannabis Use	351	0.05[a]	0.18	0.15	0.16
	Age		0.08		0.33	
	Maternal Social Class		0.23		0.43	
	Conduct Disorder Symptoms (10 yrs)		0.45		0.63	
	Cumulative Cigarettes Smoked (15.5 yrs)		0.01[a]		.03[a]	
	Cumulative Binge Drinking (15.5 yrs)		0.27		0.12	
	Cumulative Cigarettes Smoked (18 - 21 yrs)		0.33		0.20	
	Cumulative Binge Drinking (15.5 yrs)		< .01[a]		0.01[a]	
	Frequency of Marijuana Use (18 - 21 yrs)[7]		0.21		0.31	

[a]Significant at p<.05 level.

[1]Never Users (n = 224), Early Users first smoked marijuana by 16.5 years (n = 122), Late Users first smoking marijuana after 16.5 years(n = 122)

[2]Age presented in months.

[3]Based on current/most recent occupation; graded ordinall from I (professional) to IV (semiskilled) and V (unskilled manual workers).

[4]Percent likelihood child would be diagnosed as having conduct disorder by ICD-10, DSM standards; parent-informed; computer predicted.

[5]Number of times adolescent drank 5 or more times in the last 2 years.

[6]Number of times adolescent drank 6 or more times in the last 2 years.

[7]Frequency within the last year.

Table 12. Results of cannabis dose ANCOVA models predicting cortical thickness.

			Cortical Thickness			
			Bilateral Mean		CB-1 Weighted	
			M = 2.68 (.08)		M = 2.79 (.09)	
		n	p	R^2	p	R^2
Model 1: Cumulative Cannabis Dose	Cumulative Cannabis Dose	101	0.03[a]	0.09	0.028[a]	0.09
Model 2: + Age [1]	Cumulative Cannabis Dose	101	0.036[a]	0.11	0.033[a]	0.11
	Age		0.12		0.13	
Model 2: + Environment	Cumulative Cannabis Dose	82	0.015[a]	0.18	0.019[a]	0.18
	Age		0.42		0.35	
	Maternal Social Class[2]		0.44		0.43	
Model 3: + Behaviour	Cumulative Cannabis Dose	81	0.018[a]	0.17	0.023[a]	0.18
	Age		0.49		0.35	
	Maternal Social Class		0.58		0.56	
	Conduct Disorder Symptoms (10 yrs)[3]		0.91		0.79	
Model 4: + Early Substance Use	Cumulative Cannabis Dose	78	0.09	0.36	0.15	0.34
	Age		0.37		0.25	
	Maternal Social Class		0.90		0.97	
	Conduct Disorder Symptoms (10 yrs)		0.50		0.40	
	Cumulative Cigarettes Smoked (15.5 yrs)		0.04		0.06	
	Cumulative Binge Drinking (15.5 yrs)[4]		0.84		0.91	
Model 5: + Current Substance Use	Cumulative Cannabis Dose	78	0.044[a]	0.62	0.09	0.56
	Age		0.39		0.71	
	Maternal Social Class		0.54		0.74	
	Conduct Disorder Symptoms (10 yrs)		0.63		0.40	
	Cumulative Cigarettes Smoked (15.5 yrs)		0.04		0.06	
	Cumulative Binge Drinking (15.5 yrs)		0.85		0.99	
	Cumulative Cigarettes Smoked (18 - 21 yrs)		0.28		0.42	
	Frequency of Binge Drinking (18 - 21 yrs)[5]		0.03		0.08	
	Frequency of Marijuana Use (18 - 21 yrs)[6]		0.41		0.45	

[a]Significant at p<.05 level.
[1]Age presented in months.
[2]Based on current/most recent occupation; graded ordinall from I (professional) to IV (semiskilled) and V (unskilled manual workers).
[3]Percent likelihood child would be diagnosed as having conduct disorder by ICD-10, DSM standards; parent-informed; computer predicted.
[4]Number of times adolescent drank 5 or more times in the last 2 years.
[5]Number of times adolescent drank 6 or more times in the last 2 years.
[6]Frequency within the last year.

3.4 Cortical Thickness of CB1 Density Terciles

Analysis of Variance. Univariate ANOVAs were conducted using CU and CD to predict mean cortical thickness in the left hemisphere, one for each tercile of cortical regions with Low, Moderate, or High *CB1* density.

Cannabis Use. In the entire sample, adolescents' mean cortical thickness (in the left hemisphere) did not differ statistically in Low, Moderate, or High *CB1* density terciles. Figures 4, 5, and 6 illustrate the lack of difference between CU groups. We fail to reject the null hypothesis of equal means across our different Cannabis Use participant groups in all three sets of cortical regions with the low, mid and high levels of *CB1* gene expression. Results are detailed in Table 13 below.

Table 13. Results of ANOVAs predicting CT in *CB1* density terciles with Cannabis Use.

	Cannabis Use						
	M(SD)						
CB-1 Density	Never	Late	Early	df	*F*	*p*	η_p^2
Low	2.43 (.1)	2.43 (.09)	2.43 (.09)	2, 468	0.12	0.89	>.01
Mid	2.81 (.09)	2.81 (.09)	2.82 (.09)	2, 467	0.68	0.51	>.01
High	2.99 (.13)	2.97 (.13)	2.99 (.13)	2, 468	0.48	0.62	>.01

Cannabis Dose. Cannabis dose (CD) was significantly associated with cortical thickness in the set of regions with high and low (but not moderate) densities of *CB1* receptors as shown in Table 14. The relationship was more pronounced in cortical regions exhibiting the lowest levels of *CB1* receptors as compared with those having the highest levels *CB1* receptors, $F(3, 98) = 6.09$, $p = <.01$, $F(3, 98) = 3.23$, $p = .03$. In regions with Low *CB1* receptor density, 16% variance in cortical thickness could be attributed to CD in regions, compared to 9% variance in regions with a High *CB1* density.

Table 14. Results of ANOVAs predicting CT in *CB1* density terciles with Cannabis Dose.

	Cannabis Dose							
	M (SD)							
CB-1 Density	Lowest	Low	High	Highest	df	*F*	*p*	η_p^2
Low	2.47 (.01)	2.41 (.02)	2.44 (.02)	2.38 (.02)	3, 98	6.09	<.001[a]	0.16
Mid	2.85 (.01)	2.83 (.02)	2.82 (.02)	2.79 (.02)	3, 97	1.76	0.16	0.05
High	3.00 (.02)	3.01 (.02)	2.99 (.03)	2.92 (.03)	3, 98	3.23	0.03[a]	0.09

[a] Significant at p >.05 level.

Post hoc analysis using the Bonferroni correction revealed within Low *CB1* density regions, there was a significant difference in mean cortical thickness between the highest and lowest CD groups (2.38± .106 vs. 2.47± .106, $p = .001$). In addition, the difference between the mean of the Highest dose group, and the High dose group trended towards significance, $p = .07$. Regarding cortical regions with High *CB1* density, a post hoc Bonferroni test revealed the same pattern; a significant difference was found between the highest and lowest CD groups, and a difference reaching borderline significance between the Highest and High dose groups, $p = .02$, $p = .052$. Mean difference between the Highest and Lowest CD groups was approximately equal in both Low and High *CB1* density regions, with a mean difference of M = .11 and M = .10, respectively.

Figure 3. Mean cortical thickness of Low *CB1* Density Regions based on Cannabis Dose.

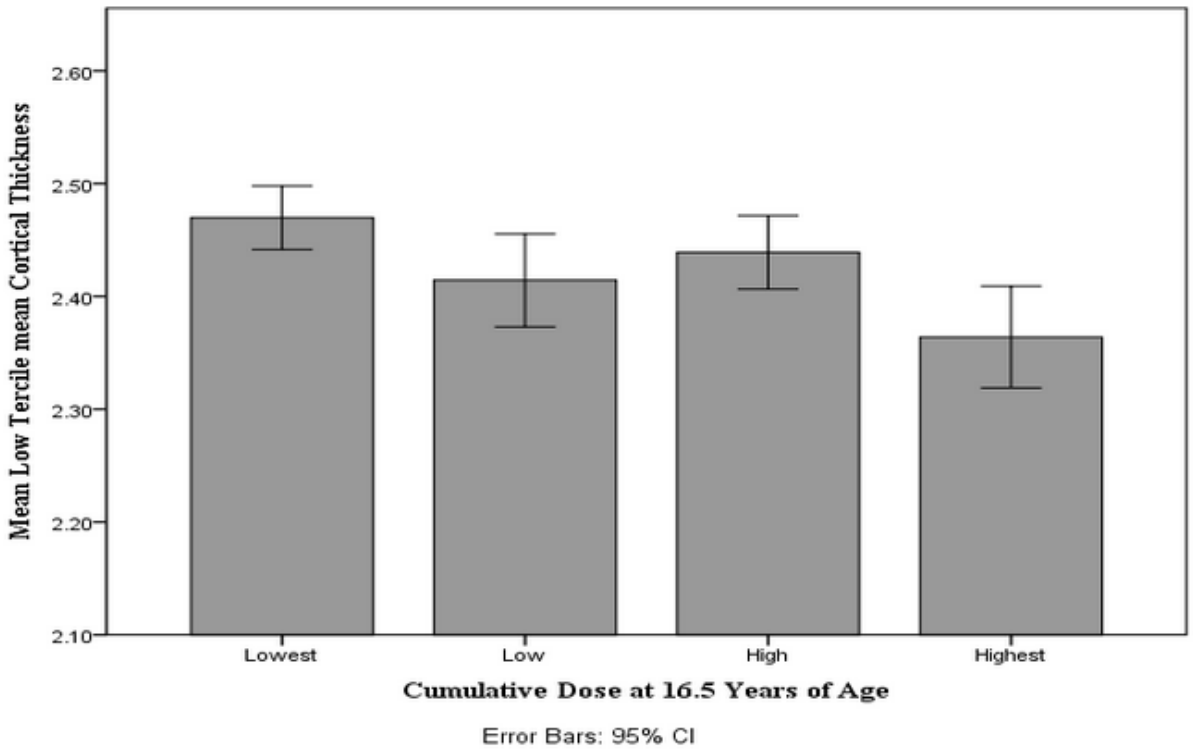


Figure 4. Mean cortical thickness of Moderate *CB1* Density Regions based on Cannabis Dose.

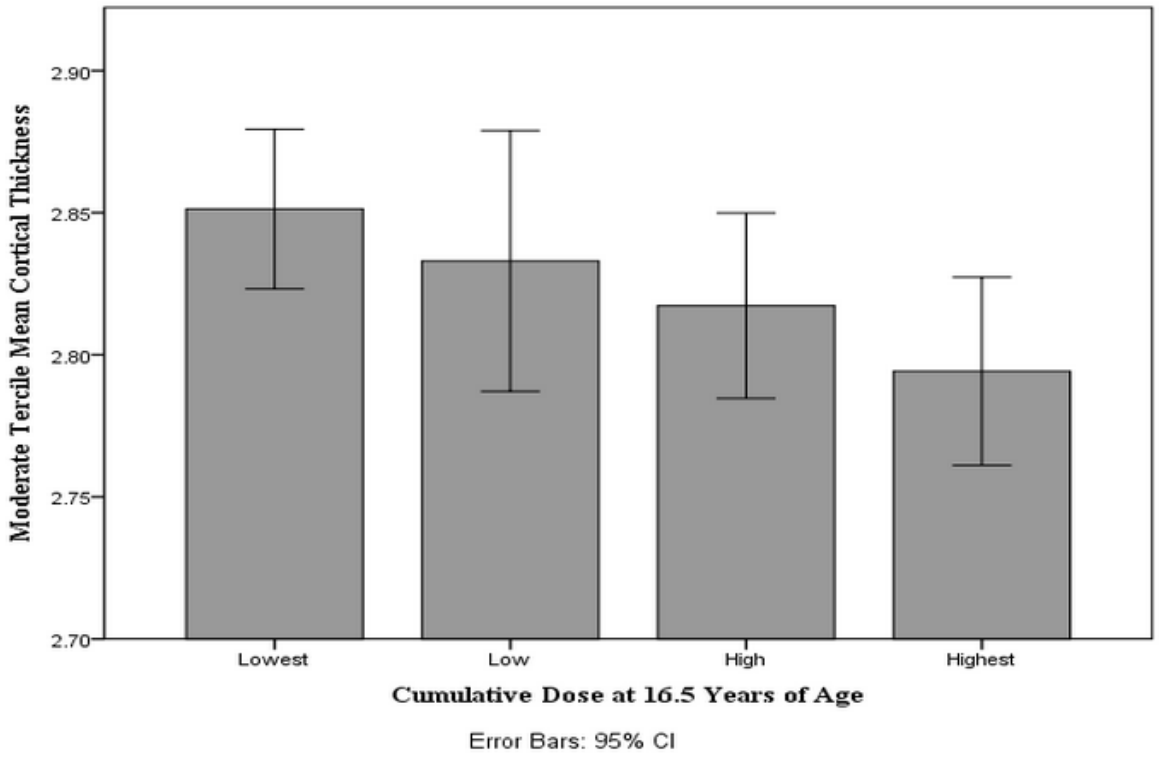
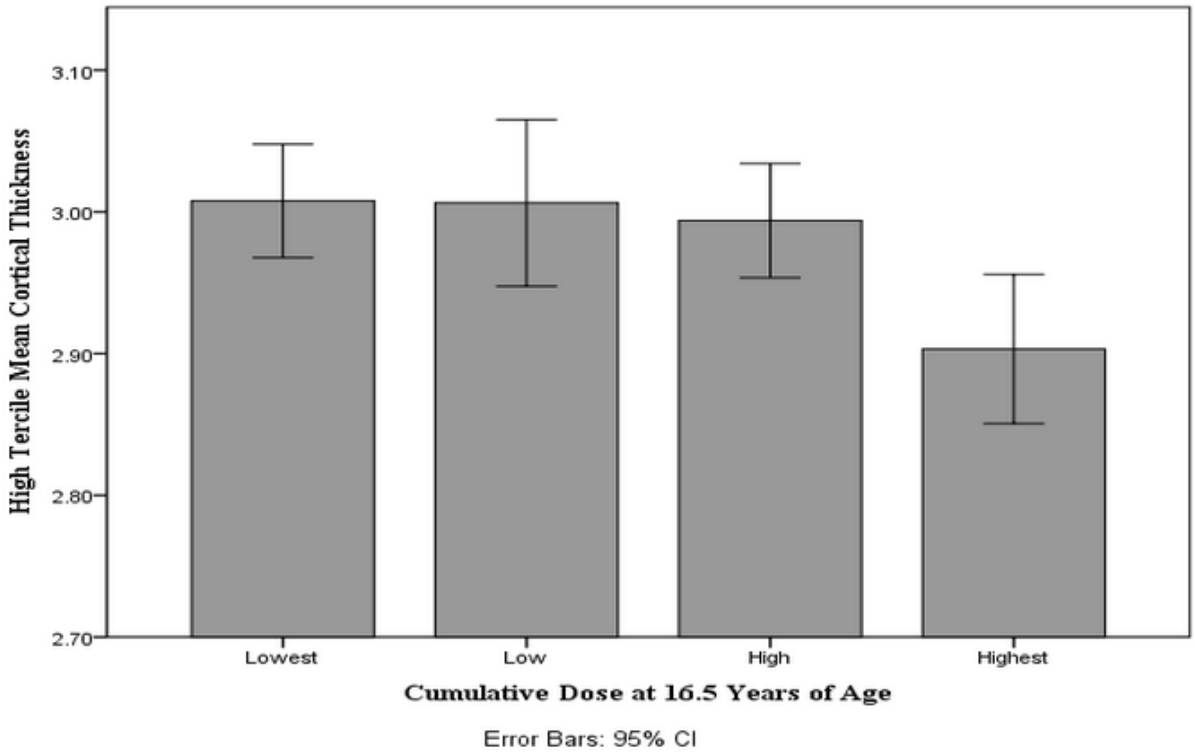


Figure 5. Mean cortical thickness of High *CB1* Density Regions based on Cannabis Dose.



General Linear Model. Based on the above evidence that groups differences between CU groups do not exist, or are not detected by our analyses, we performed multiple GLM Univariate ANCOVAs for each *CB1* density tercile using our CD subset only.

Cannabis Dose. Our results in Table 15 show that CD was a significant predictor for ANCOVA models in predicting thickness in cortical regions with Low *CB1* density and High *CB1* density, but not those with Moderate CB1 density. Additionally, CD remained significant throughout all models, surviving adjustment for early use and present use of cigarettes and alcohol in Low *CB1* density regions. For High *CB1* density regions, CD remained significant until the final model. In regions with Moderate *CB1* density, CD only approached significance in Models 2 and 3, $p = .07$, $p = .08$. Cannabis dose significantly predicted mean cortical thickness until Model 4 in high *CB1* receptor regions, whereas CD remained significant in Model 4 for low *CB1* receptor regions, $F(3, 55) = .44$, $p = .05$. Notably, in all models for Low and High *CB1* density cortical regions, CD was the most significant predictor.

Table 15. Results of ANCOVAs predicting CT in *CB1* density terciles with Cannabis Dose.

			Mean Cortical Thickness					
			High CB-1		Moderate CB-1		Low CB-1	
		n	p	R^2	p	R^2	p	R^2
Model 1: Cumulative Cannabis Dose	Cumulative Cannabis Dose	101	0.03[a]	0.09	0.16	0.05	.001[a]	0.16
Model 2: + Age [1]	Cumulative Cannabis Dose	101	0.03[a]	0.11	0.18	0.06	.001[a]	0.17
	Age		0.13		0.32		0.20	
Model 2: + Environment	Cumulative Cannabis Dose	82	0.02[a]	0.19	0.07	0.14	<.001[a]	0.23
	Age		0.22		0.62		0.58	
	Maternal Social Class[2]		0.50		0.57		0.55	
Model 3: + Behaviour	Cumulative Cannabis Dose	81	0.03[a]	0.19	0.08	0.14	.001[a]	0.23
	Age		0.18		0.68		0.69	
	Maternal Social Class		0.67		0.62		0.74	
	Conduct Disorder Symptoms (10 yrs)[3]		0.74		0.78		0.98	
Model 4: + Early Substance Use	Cumulative Cannabis Dose	78	0.04[a]	0.35	0.25	0.31	0.04[a]	0.38
	Age		0.13		0.63		0.48	
	Maternal Social Class		0.84		0.90		0.93	
	Conduct Disorder Symptoms (10 yrs)		0.57		0.47		0.72	
	Cumulative Cigarettes Smoked (15.5 yrs)		0.27		0.10		0.05	
	Cumulative Binge Drinking (15.5 yrs)[4]		0.66		0.95		0.68	
Model 5: + Current Substance Use	Cumulative Cannabis Dose	78	0.10	0.41	0.14	0.58	0.04[a]	0.58
	Age		0.26		0.26		0.32	
	Maternal Social Class		0.86		0.30		0.81	
	Conduct Disorder Symptoms (10 yrs)		0.32		0.46		0.68	
	Cumulative Cigarettes Smoked (15.5 yrs)		0.37		0.06		0.08	
	Cumulative Binge Drinking (15.5 yrs)		0.84		0.92		0.68	
	Cumulative Cigarettes Smoked (18 - 21 yrs)		0.76		0.47		0.64	
	Frequency of Binge Drinking (18 - 21 yrs)[5]		0.76		0.04		0.10	
	Frequency of Marijuana Use (18 - 21 yrs)[6]		0.89		0.50		0.18	

[a]Significant at p<.05 level.

[1]Age presented in months.

[2]Based on current/most recent occupation; graded ordinall from I (professional) to IV (semiskilled) and V (unskilled manual workers).

[3]Percent likelihood child would be diagnosed as having conduct disorder by ICD-10, DSM standards; parent-informed; computer predicted.

[4]Number of times adolescent drank 5 or more times in the last 2 years.

[5]Number of times adolescent drank 6 or more times in the last 2 years.

[6]Frequency within the last year.

Chapter 4
Discussion

4 Discussion

4.1 Conclusions

Broadly, our findings support our hypothesis that cannabis exposure during adolescence is related to cortical thickness in young adulthood. The higher number of times an individual smoked cannabis during early adolescence (before 16.5 years) is associated with a thinner cortex in young adulthood. Post hoc analyses revealed a large effect size: mean cortical thickness of participants who smoked the most cannabis in their lives (at least 60 times) had a thinner cortex as compared with those who smoked the least (less than 5 times), a difference of one standard deviation.

The relationship between cannabis dose and cortical thickness proved stable after controlling for numerous confounders, remaining significant in all but one of our ANCOVA models. From this we can infer that the observed negative correlation between cannabis dose and cortical thickness is not confounded by age, maternal SES, conduct disorder symptoms nor to cumulative lifetime cigarette smoking, frequency of binge drinking, or cannabis use in young adulthood. When adjusting for substance use variables in early adolescence (cigarettes smoked and binge drinking frequency) our cannabis dose predictor variable drops below significance (see Table 12). When cortical thickness means are adjusted for substance use variables both in early adolescence and young adulthood, cannabis dose is once again related to cortical thickness, as can be seen in Model 5 (see Table 12). Our ANCOVA model results suggest cumulative cigarette smoking in early adolescence, as well as frequency of binge drinking in young adulthood are also predictive of mean cortical thickness, demonstrating similar levels of significance as our cannabis dose predictor.

That the lowest and highest cannabis dose groups differed makes for an intuitive interpretation; specifically, that our finding a negative correlation of cortical thickness with and cumulative dose likely reflects an underlying dose response relationship in which increased cannabis dose results an increase in cortical loss or lack of cortical development. Our results suggest that a minimum of approximately 60 occasions of smoking cannabis may be necessary to result in detectable cortical loss as a global measure. Given our measure, we cannot speculate as to the effect of cumulative exposure to cannabis when onset occurs in late adolescence (after the age of 16.5) or later. Overall the findings from this study posit that the more cannabis an individual smokes during early adolescence, the more likely it is that their young adult brain will have less cortical gray matter.

While we found cumulative dose to be related to cortical thickness, we also found evidence that other metrics of cannabis use may be much less robust predictors of cortical thickness. Analysis of

variance revealed no group differences in mean cortical thickness in comparing cannabis use or cannabis use onset with our cannabis use variable. Only after adjusting for use of cigarette and alcohol use in our final two ANCOVA models did cannabis use just reach significance in predicting cortical thickness; unlike our models using cumulative dose as a predictor, in these models frequency of binge drinking in young adulthood appeared to be a more significant predictor of cortical thickness than cannabis use and accounted for more partial variance than cannabis use (see Table 11., Table 12., and Table 13.). Our results suggest that cumulative dose is the most predictive, and potentially the only predictive factor in determining cortical thickness structure in cannabis smokers.

We informed our cortical thickness measure by *CB1* receptor density as indexed by *CB1* gene expression through two different methods. Our first method of creating a weighted average of global cortical thickness based on *CB1* receptor density (where regions with higher *CB1* density had greater weight in determining the average) did not improve the performance of our cumulative dose variable in our ANCOVA models, exhibiting similar levels of significance (see Table 11. and Table 12.). Contrary to our hypothesis, a global *CB1* weighted measure of mean cortical thickness appeared to be slightly less robust in detecting differences based on cannabis exposure. For this there are several possible explanations. First, *CB1* gene expression data provided by the ABI may not be representative as only 6 donors contributed to gene expression in the left hemisphere while only 2 contributed to the right. Second, regions with high *CB1* density may be affected by one or more external factors not included in our ANCOVA models to a greater extent than cumulative dose, which increased noise and reduced the variance accounted for by our models (see Table 12.). This is based on cumulative dose being slightly more significant in predicting, and accounting for equal variance in *CB1* weighted cortical thickness in our ANOVA, before adjusting for other variables (see Table 12.). Lastly, a negative association between cumulative dose and cortical thickness may not be limited to regions with high *CB1* density as was assumed by assigning more weight to high-density regions; this final interpretation was supported by our second method of analyzing *CB1* density.

Our second method of comparing the association of cannabis exposure with cortical thickness based on relative *CB1* density yielded group differences between terciles of cortical regions with relatively high, moderate or low *CB1* gene expression. Regions with both Low and High *CB1* receptor density were negatively correlated with mean cortical thickness. Further, the observed negative correlation was more significant in regions with the lowest *CB1* density as compared with highest by an order of magnitude (see Table 15.). Relative to the amount of variance cumulative dose explains in global cortical thickness, cumulative dose explains approximately the same proportion of variance for high *CB1* density regions but accounts for almost double that in regions with low *CB1* receptor density. Within low *CB1* density regions cumulative dose was predictive in all ANCOVA models. Within high *CB1* density regions cumulative dose survived until controlling for current substance use factors in our final ANCOVA

model. Thus, cumulative dose appears to be most robust predictor of cortical thickness in regions low in *CB1* receptors. Interestingly, cumulative dose was the sole predictor of cortical thickness in regions with high and low *CB1* in our final ANCOVA model. In contrast, frequency of binge drinking in young adulthood was related to cortical thickness in regions with moderate *CB1* density and our mean cortical thickness in our final ANCOVA models. Likewise, cumulative cigarettes smoked in early adolescence was related to mean cortical thickness in regions with moderate CB1 density, only trending towards significance in our final model for low, but not high *CB1* density regions (see Table 12. and Table 15.). As we only tested this using *CB1* gene expression and cortical thickness data regarding the left hemisphere, our conclusions are limited in the left hemisphere. Overall our analyses yield evidence our previously observed association of cumulative dose with cortical thickness is heterogeneously found in the cortex, and that *CB1* receptor density differentiates whether or not a relationship is observed, with low and high density regions showing lesser grey matter. Lastly, our analyses suggest that cortical regions with the highest and lowest densities of *CB1* receptors may be vulnerable to the effects of cumulative cannabis exposure. Again, we consider several possible explanations in our interpretation of these findings. First, these analyses complement our previous finding that cumulative dose was a similar and somewhat less robust of a predictor for *CB1* weighted cortical thickness as compared with global mean cortical thickness. Regions were weighted proportionally to their *CB1* density in our *CB1* weighted cortical thickness, meaning greater influence was selectively assigned to regions with high *CB1* density. Conversely, less influence was therefore assigned to regions with low *CB1* density. In doing so, we maximized any potential association in regions with higher density and minimized that in regions with lower densities. As our analyses of cortical thickness by *CB1* density terciles revealed associations with both low and high density regions, with the stronger exhibited by low density regions, a loss of explained variance in regions with low *CB1* density is consistent with our finding a *CB1* weighted average to be a similar but less robust predictor of cortical thickness.

Given we have no MRI data prior to participants' onset of cannabis use, we have no reference of individuals' brain structure from which to compare and deduce a change over time. Therefore, we cannot assume directionality of the relationship between cannabis dose and cortical thickness and are thus unable to speak to causation. We are limited to characterizing the observed association in terms of conclusions.

4.2 Potential Mechanisms

Our finding that a greater cumulative use of cannabis in early adolescence is associated with a thinner cortex in young adulthood could reflect either a causal or noncausal relationship between cannabis use and cortical thickness. As causation cannot be attributed to cannabis smoking based on our data, we will speculate about potential causal and noncausal underlying mechanisms to which one could attribute our observed correlation. We explore two potential mechanisms through which cannabis use may catalyze

the loss, or attenuated development of cortical grey matter. Based on the discussed research, we suggest one or both may contribute to our findings, as illustrated in Figure 9. We also discuss one additional mechanism responsible for a loss of grey matter with which cannabis use may correlate, as illustrated in Figure 10.

We propose two pathways through which cannabis could result in lesser cortical thickness, both by altering neuroplasticity: psychosocial and pharmacological. Experience-related plasticity is the effect of learning experiences on morphology and synapse number, which typically increase in response to learning (Markham & Greenough, 2004); we hypothesize that cannabis use may interfere with this process in one or more ways. First, psychosocial effects of smoking cannabis may reduce the tendency to experience learning. Second, pharmacological effects of smoking cannabis may reduce the neural response to learning through either electrophysiological inhibition of neurons, or alternatively through decreasing neurotrophin levels. Further, should our observations reflect a direct effect of cannabis, at least one of the several components comprising cortical grey matter had to have been affected by the pharmacological effects of cannabis. Thus, to account for our findings we consider what component(s) of grey matter would be more likely affected.

Experience-dependent plasticity. It has been long established that both exposure to complex or enriched environments and learning specific skills can lead to structural alterations in the brain (Markham & Greenough, 2004). Enriched environments are characterized as being complex spatially and visually, incorporating daily novelty, and providing access to physical activity. Evidence suggests the process of learning and exposure to enriched environments leads to greater cortical grey matter development, and increases in cortical grey matter; this process has been referred to as experience-dependent plasticity. Importantly, experience-dependent plasticity is believed to be facilitated at least in part by neurotrophins such as BDNF and NGF.

In the 1970s, Greenough and colleagues were among the first to demonstrate that learning can induce long-lasting morphological changes either via the environment or through the acquisition of knowledge or a specific skill. Experiments have shown structural differences in response to learning through enriched environments as measured by complex environment paradigms as well as skill learning paradigms. Relative to rats reared either alone or with other rats in standard environments, rats reared in complex or enriched environments exhibit greater total brain weight (Krech, Rosenzweig, & Bennet, 1960). One of the earliest structural distinctions made about rats reared in EEs is that they exhibit thicker cortical grey matter by approximately 6.4% in the visual cortex (Diamond, Krech, & Rosenzwieg, 1964). Research has uncovered differences in dendrites, synapses, glia, axons, and vasculature unique to rats raised in enriched environments which could account for these differences in brain structure. Relative to rats raised in isolation, rats raised in enriched environments have larger dendritic fields as well as higher synaptic density per neuron (Turner & Greenough, 1985; Volkmar & Greenough, 1972). Wallace et al.

(1992) later showed dendritic changes associated with enriched environments, specifically longer dendrites, as well as greater number of dendritic branches in the visual cortex emerged after housing rats in an enriched environment for only 4 days. More oligodendrocytes (by 27 to 33%) by 30 days, and more astrocytes (by 13%) by 80 days have also been measured in rats reared in enriched environments relative to isolated controls (Szeligo & Leblond, 1977). Diamond et al. (1966) reported a 14% increase in glia in sections of the visual cortex among rats reared in enriched environments. Exposure to enriched environments has also been associated with greater total surface area of astrocytes as well as larger capillary volumes (by approximately 80%) per individual neuron (Black, Isaacs, Anderson, Alcantara, & Greenough, 1987; Sirevaag, Black, & Greenough, 1991). Rats reared in enriched environments have also been shown to have axons larger in size, and a greater number of unmyelinated axons relative to isolated controls (Juraska & Kopcik, 1988).

Regarding the experience of learning a specific skill, rats who underwent motor skill learning showed more synapses per neuron in the motor cortex, increased cortical thickness in the motor cortex, and reduced motor cortical cell density as compared with non learning controls (Anderson, Li, Alcantara, Isaacs, Black, & Greenough., 1994; 1996, 2002; Díaz et al. 1994; Kleim, Lussnig, Schwars, Comery, & Greenough, 1996). Greenough and colleagues have shown rats trained to reach for food with specific paws have larger apical dendritic fields, dendrites longer in length, a larger number of oblique branches from the apical shaft, longer length of terminal branches relative to controls, and alterations in branching complexity in corresponding regions in the motor-sensory cortex (Greenough, Dahlgren, Sagar, Gönenç, & Lukas, 1985; Greenough, Hwang, & Gorman, 1985; Withers and Greenough; 1989). Response to motor skill learning has been shown to have a greater number of synapses per Purkinje cell in the cerebellum, as well as in tissue of approximately the volume of one Purkinje cell relative to inactive and exercised controls (Black et al., 1990; Kleim et al., 1998). Greater synaptic density, greater glial cell volume, and more astrocytes per Purkinje cell in the cerebellar cortex as compared with controls (Kleim et al., 2007). Employment of physical activity control groups have been able to dissociate observed differences attributed to enriched environments and skill learning from physical activity either completely or partially. For example, Black et al., (1990) dissociated observed effects of learning and physical activity, showing only rats that underwent motor skill learning rats from greater numbers of synapses, whereas only rats undergoing physical activity showed evidence of angiogenesis, having a greater density of capillaries. Similarly, enriched environments appear to significantly increase cortical weight (cortical/subcortical ratios), whereas exercise alone produced insignificant increases in cortical weight relative to an inactive control groups - hypothesized to partially contribute to the observed effect of enriched environments (Huntley and Newton 1972). While an exercise control group was shown to have a greater density of capillaries, they did not exhibit the same increase in synapses per neuron in the cerebellum as the motor skill learning group (Isaacs et al. 1992). Anderson et al. (1994) demonstrated that

motor skill learning but not exercise induces genesis of synapses and astrocytes. Rats in the learning group but not exercise group exhibited greater volume of molecular layer and glia per Purkinje cell. Glial volumes did not differ per synapse or per capillary between rats, showing glial changes exhibited in learning appear related to synaptogenesis and not vascular alterations. Wallace and colleagues' observations that rats housed in enriched environment for only 4 days showed greater neuropil but not vasculature than controls further also offers support that the response to learning (via enriched environments) is genesis of neurons and astrocytes, and that increases in vasculature appear to follow (Wallace, Withers, Farnand, Lobingier, & McCleery, 2011). Altogether this suggests that both neuropil and vasculature components of the cortex are altered by the experience of learning via the environment or experience, though alterations in neuropil appear to precede vasculature in response to learning, while changes in vasculature appear to be related to activity (i.e. activation via physical activity) rather than learning (Markham & Greenough, 2004).

In humans, morphological changes directly attributable to learning was first reported by Draganski et al. in 2004, reporting an increase in grey matter volume of in the occipital-temporal cortex in response to a learning paradigm in which novices learned to juggle 3 balls. Magnetic resonance imaging (MRI) was used to measure these structural differences, showing volume had increased by 3%; a follow up scan 3 months later revealed the increase had fallen to 2%. Draganski and colleagues have since shown that learning abstract information also induces structural differences detectable using MRI data. Using voxel based morphometry (VBM), Draganski et al. (2006) compared brain scans of student before, during, and 3 months after studying for a medical exam. Whole brain grey matter increases were found in the posterior and lateral parietal cortex both during and after study period, while initial increases in posterior hippocampal grey matter were found during the study period but greater increases were found 3 months after (Draganski et al., 2006). Recent research has contributed to understanding structural changes in response to learning a second language. Conscript interpreters (without prior experience) were scanned before and after 3 months of language studies, showing increases of hippocampal volume and of left middle frontal gyrus, inferior frontal gyrus, and superior temporal gyrus cortical thickness (Mårtensson et al., 2012). Interestingly, some differences could differentiate those with higher and lower proficiency for acquisition of the language; specifically, proficiency was associated with alterations in the right hippocampus and the left superior temporal gyrus, while those displaying difficulty in language acquisition showed larger increases in the middle frontal gyrus. Comparisons of MRI data before and after 5 months of learning German, native English speakers displayed alterations in the left inferior frontal gyrus, and the increase of gray matter but not absolute grey matter was associated with new language proficiency (Stein et al., 2012).

***Grey matter composition*.** The components of cerebral cortex in mice can be proportionally broken down into the following cellular constituents for every 1 cubic millimeter: axons make up

approximately 29.3%, dendrites compromise 30.2%, dendritic spines contribute 12.06%, glia account for 9.5%, while cell bodies and blood vessels take up 13.8%, leaving 5.2% of extracellular space (Braitenberg, 2001). As one may expect to detect changes more likely in cortical components making up the greatest proportional volume, we hypothesize that lesser cortical thickness in participants who smoked cannabis the most represents of a volumetric loss in neuropil (axons and dendrites combined), which accounts for 60% of cortex (Braitenberg & Schuz, 1998).

4.2.1 Psychosocial Pathway

In our first proposed pathway, we conceptualize school as a socially programmed analogue of enriched environments used in animal experiments, as well as an environment that facilitates learning specific skills. We hypothesize early school withdrawal would lead to relatively less exposure to learning experiences, consequently reflected at the neural level as relatively less cortical grey matter as compared with adolescents remaining in school (see Figure 9. below).

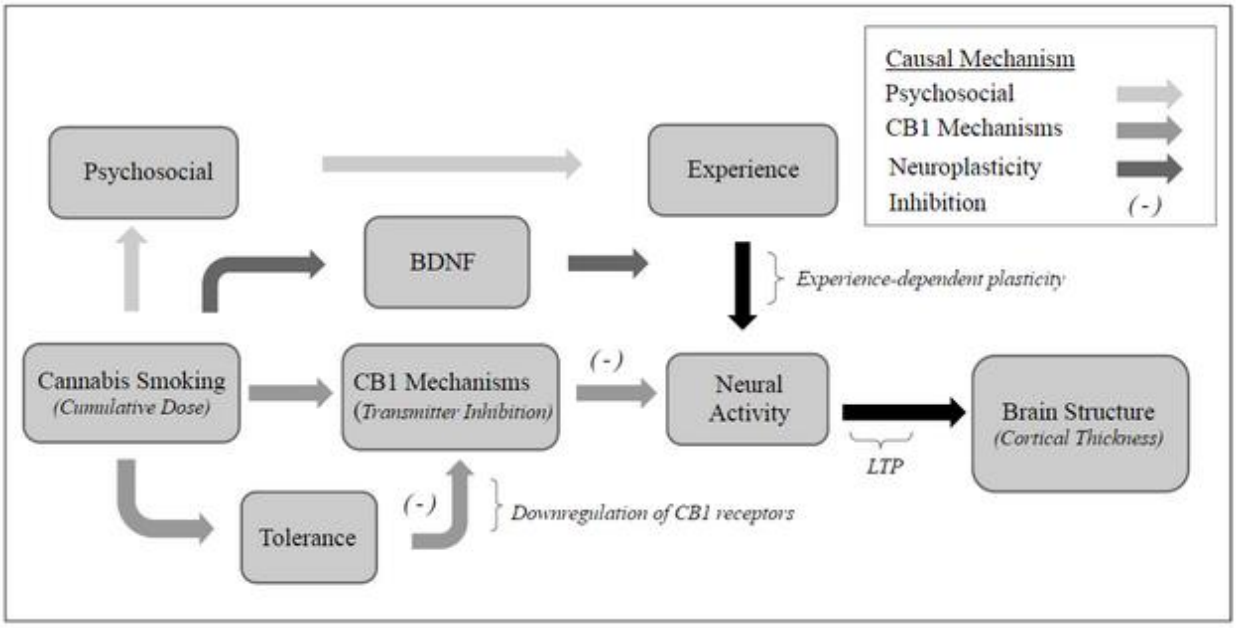
Cannabis use predisposes individuals to achieve lesser educational, correlating positively with, and predicting school dropout (Heron et al., 2013; Legleye et al., 2009; Townsend, Flisher, & King, 2007; Van Ours & Williams, 2009). An association between cannabis use and lesser educational achievement has been consistently demonstrated in previous studies. Importantly, cannabis use appears predictive of school dropout and poorer performance even after statistically controlling for various individual differences prior to cannabis use onset. In 1997, Swaim, Beauvais, Chavez and Oetting compared cannabis use among White non-Hispanic, Mexican American, and Native American students and dropouts, finding incidence of cannabis use to be between 1.2 to 6.4 times greater among dropouts across all racial/ethnic groups. Yamada et al. (2000) showed cannabis users were less likely to graduate high school after adjusting for age of dropout and use of other substances. When looking at schooling in terms of the number of years completed, Chatterji (2006) found cannabis use during high school to be associated lesser educational achievement. In 2008, Fergusson and Boden showed in a New Zealand based birth cohort that higher levels of cannabis use between 14 to 21 years of age predicted lower levels of educational attainment by age 25 (based on degree completion); the observed bivariate association held true after adjusting for socioeconomic background, family functioning, child abuse, childhood and adolescent adjustment, early adolescent academic achievement, comorbid mental disorders and substance use. Horwood et al., (2010) analyze data from three Australian cohort studies, finding highly significant associations between age of onset and all 3 educational milestones measured (high school graduation, university enrolment, university degree) when looking at data independently and pooled. Both before and after controlling for various confounders, milestone attainment was highest among those who had not used cannabis by the age of 18 and lowest among those who had used cannabis before age 15. In their review paper, Lynskey and Hall (2000) concluded cannabis use is a stable predictor of achieving less

education based on longitudinal studies controlling for various individual differences based on 8 longitudinal studies of cannabis use and educational outcomes. According to Lynskey and Hall (2000), a study by Fergusson et al. (1996) demonstrated the most thorough control of confounders. Ferguson and colleagues (1996) followed approximately 1000 adolescents from birth, finding 22.5% of participants who had smoked cannabis by the age of 15 had dropped out of high school before the age of 16 as compared with only 3.5% of those who had not smoked by 15. While Fergusson et al. (1996) showed that early cannabis users differed relative to users prior to substance use onset, exhibiting poorer mental health, poorer academic performance, more delinquency and more family dysfunction, statistical adjustment for these differences did not render the relationship insignificant; early cannabis users were still 3.1 times more likely to have left school by 16 after correction. After controlling for cognitive skill, socioeconomic status, and education expectations, Tanner et al. (1999) found cannabis use measured between 14 and 17 years was still predictive of school dropout, failure to graduate both high school and college in cohort data from the National Longitudinal Study of Youth. In conducting a longitudinal study of participants from the ages of 16 to 18 years old, Bray, Zarkin, Ringwalt and Qi (2000) reported a positive correlation between cannabis use and dropping out of high school, with those having initiated cannabis smoking approximately 2.3 times more likely to drop out than their non-using peers. A prospective study with over 4500 students by McCaffrey, Paluca, Han, and Ellickson (2010) found a positive association of cannabis and high school dropout, finding cannabis users 5.6 times more likely to dropout as compared with non users. After adjusting for differences existing at baseline (7th grade) the higher probability of dropping out for cannabis users dropped to 2.4 times. Interestingly, this remaining difference dropped below significance when cigarette smoking was also accounted for in the model. Lynskey and Hall (2000) noted that associations had previously been found between increased cannabis use and lower grade point average, less satisfaction with school, and negative attitudes towards school. This suggests cannabis use may leads to subjective disengagement from school that precede the objective measure of dropout, and may be present even in cannabis users who do not reach the extreme case of dropping out.

In following with the above literature, ALSPAC participants who had used cannabis the most (among those with early onset use) might have disengaged from the learning environment of school relative to non users, and to a greater extent than other early cannabis users. Consequently, less learning experiences would have yielded lesser experience-dependent plasticity in the heaviest cannabis users, translating into a relatively thinner cortex as we observed. This hypothesis could be tested by obtaining data pertaining to school dropout or number of years of schooling at the time of scan (retrospectively) and comparing cannabis users based on dropout status or years of education. We could determine if years of schooling moderates the negative correlation between CD and cortical thickness, though with our small number of participants in our highest CD group we could realistically have little of drop outs. In order to

capture measures of disengagement and lack of participation lesser than dropping out, number of classes missed, number of courses taken, and extracurricular activities could be used to produce an aggregate and scalar measure of school participation for comparison. If a data set did have enough participants to demonstrate sufficient power it would be important to control for premorbid (before cannabis use onset) levels of IQ, SES, and other variables correlating with academic achievement which may confound the relationship of cortical thickness and dropping out of school given the mentioned research demonstrating poorer academic performance often predates cannabis use (Lynskey & Hall, 2000).

Figure 6. Potential causal mechanisms by which cannabis may reduce cortical thickness.



4.2.2 Pharmacological Pathway

As a psychoactive drug, cannabis induces pharmacological changes in the brain upon its use. We explore cannabis' effect on neurotrophin levels and neurotransmitter signalling, and how both effects could potentially lead to a thinner cortex with cannabis use.

Neurotrophins and learning. As reviewed above, research has shown the experience of learning to cause changes in grey matter. The process by which learning is translated structurally in the brain is understood to be long term potentiation (LTP), or the strengthening of synapses in response to their various firing patterns; this capacity for change in synaptic strength is considered to be neural plasticity (Huang & Reichardt, 2001; Lim, Lim, & Federoff, 2003: Tanaka et al., 2008). Importantly, LTP is associated with enlargement of dendritic spines (one of these Huang & Reichardt, 2001; Lim et al., 2003: Tanaka et al., 2008).

Neurotrophins such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are proteins involved in differentiation, functioning, growth, and survival of new and existing neurons, making them integral to brain development and plasticity (Angelucci et al., 2008; Bramham & Messaoudi, 2005; Huang & Reichardt, 2001). Elucidated primarily by studies conducted using hippocampal tissues, BDNF mediates synaptic plasticity (Bramham & Messaoudi, 2005; Huang & Reichardt, 2001; Leal, Comprido, & Duarte, 2014; Lim et al., 2003: Tanaka et al., 2008). BDNF facilitates LTP in both a permissive and instructive manner in that it enables and potentiates LTP, respectively (Bramham & Messaoudi, 2005; Gottman et al., 2009) For example, BDNF enhanced LTP in the visual cortex and hippocampal synapses. Corroborating this research are animal studies show memory deficits, success rate of induced LTP, magnitude of successfully induced LTP, and an absence of long-lasting protein synthesis-dependent LTP in BDNF knockout mice (Huang & Reichardt, 2001; Korte & Bonhoeffer, 1997); success of LTP was reduced from 90% to 30% of induction attempts (Korte & Bonhoeffer, 1997). Exogenous BDNF was also found almost completely reverse deficits in LTP seen in BDNF knockout mice (Huang & Reichardt, 2001; Korte & Bonhoeffer, 1997; Leal et al., 2014).

Importantly, BDNF is necessary and sufficient to induce structural alterations associated with synaptic plasticity, such as long-lasting changes of dendritic spine morphology (Tanaka et al., 2008). An experiment involving synaptic stimulation of hippocampal neurons determined BDNF action specifically (as well as protein synthesis) was required for the gradual enlargement of individual dendritic spine heads in CA1 pyramidal neurons required endogenous (Tanaka et al., 2008). BDNF was also found to increases synaptic spine density (one of Huang & Reichardt, 2001; Korte & Bonhoeffer, 1997; Leal et al., 2014). In further support of BDNF as a gatekeeper of activity-dependent plasticity, attenuated amplitudes of training-dependent increases in motor evoked potentials, along with corresponding cortical reorganization, are observed in individuals with the less efficient polymorphism (val66met) of the BDNF gene (Kleim, et al., 2006).

Levels of neurotrophins. Research suggests that exposure to cannabis or its psychoactive component (THC) affects neurotrophin levels. In 2008, Angelucci and colleagues reported lower serum levels of NGF, but not BDNF, in cannabis-dependent participants as compared with healthy controls. D'Souza and colleagues (2009) later found BDNF to differ in current light cannabis users (defined as a minimum of 10 exposures in last month and 100 over lifetime, with positive urine test or cannabis, and current CUD) relative to healthy controls with non-current (based on a negative urine toxicological test) and never-chronic use. D'Souza's (2009) conclusion of reduced BDNF was based on 6 samples per subject at baseline, during placebo and THC administration (0.0286 mg/kg over 20 minutes), and afterwards. D'Souza's (2009) design was unique, enabling inference regarding differences between cannabis users and non-users at baseline (demonstrating long term effects of cannabis exposure) and in

response to acute administration of cannabis. Cannabis users had lower basal BDNF at baseline as well as at all proceeding time points throughout the experiment, demonstrating reduced BDNF at baseline as likely a consequence long-term cannabis use. Where non-users showed significant increases in BDNF in response to THC treatment, users did not, showing attenuated responses to acute effects in current users; authors attributed the observed lower sensitivity of BDNF levels in cannabis users to lower basal levels of BDNF, though other experiments would be necessary to rule out desensitization as a possibly independent consequence of current cannabis use. (D'Souza et al., 2009). Importantly, concentrations of BDNF in blood have been shown to correlate with levels of BDNF in the brain (Klein et al., 2011). In rats, Maj and colleagues (2007) similarly showed long-term reductions of BDNF in the hippocampus and frontal cortex in response to long-term, prenatal exposure (0.5 mg/kg per day from gestation day 5 - 20) to *CB1* agonist treatment.

In contrast, some studies have reported increases in neurotrophins, reporting up-regulation of BDNF mRNA expression, and increases of neurotrophin levels (BDNF and PTN) by between 10% and 10 fold (Butovsky et al., 2005; Fishbein et al., 2012; Mailleux et al., 1994). However, these studies differed from those finding reduced neurotrophins in important ways; these studies examined neurotrophin level change in response to a single administration of THC, and were all conducted using rodents, postnatally. Studies reporting reduced neurotrophins were based on exposure to cannabinoids in humans or in rats, prenatally, as well as in response to long term exposure, or acute exposure in long term users of cannabis; in this way these studies, particularly that of D'Souza and colleagues (2009) were more analogous to our own, and therefore more relevant in terms of interpreting our findings.

Altogether, these studies suggest BDNF is altered by cannabis exposure, and further that this response may vary depending on the length (acute or long-term), pattern (chronic or intermittent), and timing (prenatal or postnatal) of exposure. The above literature evidences BDNF increase following chronic, short term exposure and acute exposure in cannabis-naive subjects (Butovsky et al., 2005; D'Souza et al., 2009; Mailleux et al., 1994). Decreases in BDNF appear to be elicited by long-term as well as prenatal exposures (D'Souza et al., 2009; Maj et al., 2007). Supporting this research is a recent study by Navakkode and Korte (2014) showing WIN55, 212-2, a *CB1* receptor agonist similar to yet more potent than THC, impaired long-term potentiation (LTP) through altering protein synthesis resulting in an increase in production of plasticity-related proteins (PRPs) in CA1 hippocampal neurons in rats. Authors hypothesized that several PRPs may be increased following *CB1* receptor binding to agonists, including BDNF. Disabling of LTP was shown to be induced by a higher dose (2 uM) but not lower dose (1 uM), which is cohesive with our findings in that evidence of alterations in neuroplasticity (inhibited LTP, indicative of an increase in PRPs) was shown to be dose-dependent.

Based on research showing lower basal levels of neurotrophins in cannabis-dependent, and current cannabis users with over 100 lifetime cannabis exposures, our highest CD group may have

behaved similarly as they had to have smoked cannabis at least 61 times (Angelucci et al., 2008; D'Souza et al., 2009). Since BDNF is known to facilitate neuroplasticity, lower BDNF may have resulted in a blunted neurochemical capacity for experience-related plasticity. Consequently, even if the quantity of learning experiences were equal across cannabis users, the translation of learning experiences to increased grey matter development would have been attenuated in the highest CD group, resulting in an overall thinner cortex as we observed. We hypothesize that early and, or that long-term exposure to cannabis could have lowered levels of BDNF in our high CD group; consequent of depleted BDNF, this group would have a deficit in LTP inhibiting the strengthening of synapses and subsequent growth of dendritic branches in response to experience which over time could manifest as an overall thinner cortex.

This hypothesis could be tested by investigating whether BDNF influences our observed association of cannabis use with cortical thickness. Without direct measures of BDNF, this could be indirectly tested through Mendelian randomization (a method that capitalizes on random assortment of genetic variants within a population to infer causality) in our sample since genetic data were also collected (Sheehan, Didelez, Burton, & Tobin, 2008). Given the functional Val66Met polymorphism for BDNF gene known to affect activity-dependent release of BDNF, one could compare those with more (Val/Val) or less efficient (Val/Met, Met/Met) genetic variant among those who exposed to cannabis (Egan et al., 2003; Lotfipour et al., 2009). A previous study demonstrated that a correlation between number of drugs tried and thickness of the orbitofrontal cortex in adolescents was only present in carriers of the efficient Val66Met polymorphism (Lotfipour et al., 2009). We hypothesize that our observed association between CD and cortical thickness would be moderated by participants' BDNF-related variant. If this were the case, it would provide strong evidence for the role of neurotrophin-based plasticity as the means by which cannabis exposure affects cortical structure.

Release of neurotransmitters . Pharmacological effects of cannabis in the brain are exerted vis-a-vis the main bioactive component of cannabis (THC) binding to cannabinoid 1 (CB1) receptors, the most abundant $G_{i/o}$ protein coupled receptor in the brain; THC is a partial agonist of CB1 receptors (Burkey, Quock, Consroe, Roeske, & Yamamura, 1997; Sim, Hampson, Deadwyler, & Childers, 1996). The vast majority of cannabinoid receptors in the brain are CB1 receptors, in contrast to the peripheral nervous system where there are primarily CB2 receptors (Mackie, 2008; Pertwee, 1997; Svíženská, Dubový, & Šulcová, 2008). In situ hybridization (ISH) and immunohistochemistry techniques have yielded insights as to the cellular and subcellular localization of CB1 receptors and patterns in their distribution.

Typically CB1 receptors are situated presynaptically on axon terminals of central and peripheral neurons; more rarely, they are postsynaptically located on neurons as well as on glia (Atakan, 2012; Katona et al., 2001; Mackie, 2008; Pertwee, 2006). Short and long term inhibition can be induced by brief depolarization or tetanic stimulation (Domenici et al., 2006; Kawamura et al., 2006). Neurons tend to express CB1 receptors at high or low levels, and expression appears to differ based on the subpopulations

of neurons (Marsicano & Lutz, 1999). Depending on the brain region, two distinct patterns of CB1 receptor distribution have emerged; uniformly expression, in large concentrations of one cell type, or nonuniformly, when more than one cell type shows high expression (Kano, Ohno-Shosaku, Hashimotodani, Uchigashima, & Watanabe, 2009; Marsicano & Lutz, 1999). Overall, and particularly in regions with nonuniform expression, high expression is typically shown by inhibitory GABAergic interneurons (basket cells), while low expression is found on many excitatory principal projection neurons (Kano 2009; Marsicano & Lutz, 1999).

Functionally, CB1 receptors alter transmitter release from axonal terminals of neurons on which they are located (Kano et al., 2009). Widespread distribution of CB1 receptors facilitates their functional regulation of synaptic activity throughout the brain (Kano et al., 2009; Szabo et al., 2014). Predominantly CB1 receptors have an inhibitory effect on synaptic activity, most often presynaptically reducing transmitter release upon activation (Atakan, 2012; Hill et al., 2007; Kano et al., 2009; Svíženská et al., 2008). Inhibition of neurotransmission was first evidenced in the 1970s with the observed suppression of an acetylcholine-dependent muscle twitch in guinea pigs after cannabinoid exposure (Gil et al., 1970). Inhibition has since been directly measured as reduced synaptic currents through intracellular recordings. Reductions of spontaneous excitatory postsynaptic current (sEPSCs) size (by ~50%), frequency, and amplitude, as well as suppressed inhibitory postsynaptic currents (IPSCs) have all been reported (Hájos et al., 2000; Hill et al., 2007; Katona et al., 2001; Morisset & Urban, 2001; Shen, Piser, Seybold, & Thayer, 1996; Sullivan, 1999). Over 40 experiments demonstrating cannabinoid receptor mediated inhibition of neurotransmitter release were reviewed in detail by Schlicker and Kathmann (2001), four of which used human tissue models. Transmitter release was measured in superfused tissue samples, or inferred by end organ response (e.g. postsynaptic currents, muscle contraction, alteration of blood pressure or heart rate). Inhibition of noradrenaline, acetylcholine, GABA, glutamate, dopamine, serotonin, and ATP have been experimentally demonstrated in numerous tissues and brain regions (Gifford, Samiian, Gatley, & Ashby Jr.,1997; Shen et al., 1996; Schlicker et al.,1997; Schlicker & Kathmann, 2001).The instrumental role of CB1 receptors in transmitter inhibition is supported by attenuated or abolished of the induced inhibitory response by CB1 receptor antagonists, in CB1 receptor knockdown in rats, and in CB1 knockout mice (Schlicker & Kathmann, 2001).

Though not yet fully elucidated, electrophysiological studies support several transduction mechanisms to be at least partially responsible for transmitter inhibition (Chiarlone et al., 2014; Howlett et al., 2004; Svíženská et al., 2008; Turu & Hunyady, 2010). Cannabinoid 1 receptors, as $G_{i/o}$ protein coupled receptors, are paired negatively with adenylate cyclase (and consequently cAMP) and voltage-gated calcium channels, resulting in their inhibition (Howlett, 1984; Svíženská et al., 2008). In contrast, CB1 receptors are positively coupled with mitogen-activated protein kinase (MAP) kinase and potassium channels, resulting in their stimulation (Howlett et al., 2004; Schlicker & Kathmann, 2001; Svíženská et

al., 2008; Turu & Hunyady, 2010). Inhibition of acetylcholine by cannabinoids is suppressed by a chemical stimulant of cAMP synthesis (forskolin), a lipophilic analogue of cAMP, and an inhibitor of cAMP degradation (isobutylmethylxanthine), suggesting reduction of cAMP accumulation due to decreased adenylyl cyclase to be instrumental. In another study, the cAMP stimulant (forskolin) failed to attenuate inhibition, suggesting reductions in adenylyl cyclase and consequently cAMP cannot account for transmitter inhibition wholly, or across all brain regions.). Similarly, blockers of potassium and GIRK channels also only partly attenuated cannabinoid induced inhibition in mouse nucleus accumbens, but not the hippocampus, suggesting mechanisms responsible for inhibition may vary according to brain region. Cannabinoids attenuate neurotransmitter release by reducing the duration of presynaptic action potential and by limiting calcium entry into the cell via synaptic vesicles (Svíženská et al., 2008). Glutamate release parallels suppression of calcium entry into cerebellar parallel fiber axon terminals (Kreitzer and Regehr, 2001; Zhang and Linden, 2009). Further, electrophysiological recordings and two-photon imaging show calcium voltage CB1 activation reduces GABA release through inhibiting N-type voltage-gated calcium channel function in hippocampal slices (Szabo et al., 2014).

The overall effect of CB1 activation in a given brain region is dependent upon which specific neurons the receptors are located. Immunohistochemical staining revealed that CB1 receptors are expressed on neuronal cell bodies, dendrites, and axons, and on different types of neurons, such as pyramidal cells in the hippocampus, Purkinje cells in the cerebellum, and medium spiny neurons in the striatum (Kano et al., 2009; Marsciano & Lutz, 1999; Tsou, Brown, Sanudo-Pena, Mackie, & Walker, 1997). Inhibitory GABAergic and excitatory glutamatergic neurons both express CB1 receptors (Chiarlone et al., 2014; Domenici et al., 2006; Hill et al., 2007; Katona et al., 2001; Kawamura et al., 2006). Localization of CB1 receptors on different types of neurons differentiate observed effects, such as the neuroprotective effects elicited by endocannabinoids activation of terminals on glutamatergic but not GABAergic neurons (Chiarlone et al., 2014).

Higher concentrations of CB1 receptors can be found within inhibitory as compared with excitatory synapses, with a higher ratio of CB1 receptors on GABAergic terminals as compared with glutamatergic terminals is consistent across many brain regions (Domenici et al. 2006; Kano et al., 2009; Katona et al., 1999, 2001; Kawamura et al. 2006; Marsicano et al. 2003; Tsou et al., 1997). Across forebrain regions, CB1 receptors are most often expressed on terminals of GABAergic interneurons (Freund, Katona, & Piomelli, 2003; Katona et al., 1999, 2001; Marsicano & Lutz, 1999). Double-labeling studies further elucidated CB1 receptors are predominantly (~70 - 80%) found on (neuropeptide) cholecystokinin-positive, and (calcium-binding protein) parvalbumin-negative GABAergic interneurons (Freund et al., 2003; Katona et al., 1999, 2001; Marsicano & Lutz, 1999). In hippocampal networks, approximately 85.6% of expression occurred on CCK basket cells-like interneurons, making up ~97% of all CCK interneurons, compared to only ~5% of PV basket cells. Altogether, studies suggest that in the

hippocampus and amygdala, where there is some of the highest CB1 expression, the primary function is inhibition of GABA release of surrounding somata and proximal dendrites of pyramidal neurons through axon terminals of CCK positive basket-cell interneurons (Katona et al., 1999, 2001). Suppression of GABA-mediated inhibition of hippocampal pyramidal cells from the degree and duration of pyramidal cell depolarization has been characterized as depolarization-induced suppression of inhibition (Howlett, 2004). The role of CB1 receptors on excitatory glutamatergic terminals is less understood, as their existence was determined by later studies (Hajos et al., 2001; Hill et al., 2007; Kano et al., 2009). Glutamatergic inhibition in the forebrain of GABAergic CB1 receptor knockout mice demonstrated CB1 modulation via glutamatergic axon terminals on principal neurons controls, contributing to evidence of presynaptic CB1 modulation of excitatory transmission in the forebrain (Domenici et al., 2006). Approximately 49% of (excitatory) pyramidal neurons housed CB1 receptors in the rat neocortex (Hill et al., 2007).

Inhibition of transmitter release from either inhibitory or excitatory neurons can induce increase or decrease in synaptic activity and therefore result in either a net effect of inhibition or disinhibition in a particular brain region (Atakan, 2012; Mackie, 2008). We propose CB1 receptor activation through cannabis smoking could induce cellular mechanisms resulting in lesser cortical thickness in at least two ways. First, inhibition of synaptic transmission at excitatory glutamatergic synapses may have prevented morphological changes that would have otherwise taken place. Specifically, attenuation of glutamate release would produce a net inhibitory effect, reducing synaptic currents below the threshold necessary for long-term potentiation. Consequently, dendritic branches would fail to grow thicker. Further, CB1 induced reduction of intracellular cAMP accumulation would attenuate increases in the number of synapses and synaptic boutons. Increases in cAMP lead to long-term morphological changes such as increases of synaptic boutons, and forskolin is an activator of adenylyl cyclase known to catalyze formation of new synapses by increasing cAMP production. Importantly, cannabinoids have previously been shown to suppress formation of synapses in response to forskolin (Kim & Thayer, 2001). Long-term potentiation of the region tested is a cAMP dependent process; given findings that synapse formation was blocked by forskolin but not a (membrane permeating) cAMP analog supports inhibition of cAMP production (rather than glutamatergic inhibition) as the mechanism through which cannabinoids block the formation of synapses (Kim & Thayer, 2001). Over time, a lack of growth could in this way reduce the normal trajectory of growth and lead to less cortical grey-matter.

Second, long term exposure to cannabinoids may cause a decrease in cannabinoid levels and, or functioning of CB1 receptors which may interfere with the normal response to experience and neuroplasticity; for example by interfering with the capacity for experience-dependent neuroplasticity to take place. Reversible downregulation of CB1 receptors after chronic exposure to cannabinoids has been reported in rodents and humans (Fan, Tao, Abood, & Martin, 1996; Hirvonen et al., 2012; Sim et al.,

1996). Reviewed by Howlett (2004), downregulation of CB1 receptors was most commonly reported, though increases and no change having also been reported. A 50% reduction in receptor binding in the rat brain has been shown after chronic exposure to THC in vivo; importantly, no changes were found in intracellular levels of adenyl cyclase, suggesting the exhibited tolerance was due to changes in receptor reserve (Fan et al., 1996). Another study showed desensitization by up to 70% of cannabinoid activated G-proteins in response to chronic but not acute exposure to THC (Sim et al., 1996). In humans, downregulation of CB1 receptors correlated with years of cannabis smoking in chronic daily smokers; receptor levels were shown to return to normal using PET imaging after monitored abstinence (Hirvonen et al., 2012). In this instance, cannabinoid receptors may have been downregulated among the heaviest of early cannabis users in our sample. As studies report, CB1 receptors are most often located on inhibitory GABAergic neurons. Therefore, typically, cannabinoids attenuate the release of GABA which has an inhibitory effect, resulting in a net effect of disinhibition. Upon downregulation of CB1 receptors, this disinhibition would be reduced, resulting in more levels of inhibition than normal; a net effect of inhibition may dampen synaptic transmission and fall short of that necessary to foster LTP.

Either of these possible mechanisms could be compounded by developmental timing, as was shown to mediate the inhibitory effect of cannabinoids on excitatory synaptic transmission in rats. In hippocampal neurons of neonatal (10–13 days old) rats, the single population spike and the field EPSP of hippocampal neurons were reduced by 67% and 28%, respectively, whereas no effect on population spikes were seen those of young adult (4–6 weeks old) rats (Al-Hayani & Davies, 2000). It is possible we observed a difference in cortical thickness because cannabis dose was compared among early cannabis users only, with an onset before the age of 16.

4.2.3 Hormonal Pathway

Given the biosocial context of our data, another indirect mechanism could account for our findings. Data were collected from our sample of participants, all of whom were male and undergoing developmental processes as part of adolescence, a period encompassing biological and behavioural changes including various sexual dimorphisms. It is possible the association we observed is spurious in that lesser cortical thickness and greater cumulative cannabis dose may not be causally related and instead correlate due to both being consequences of pubertal maturation in males vis-à-vis testosterone.

Research has uncovered various sexual dimorphisms in brain structure. Males' and females' brains differ in both size and content. Males exhibit larger total brain volume, greater absolute grey and white matter, and greater relative white matter as compared with females, while females exhibit greater relative white matter white matter volumes (Allen, Damasio, Grabowski, Bruss, & Zhang, 2003; Bramen et al., 2012; De Bellis et al., 2001; Paus et al., 2010). Over the course of adolescence, both sexes alike show age-related reductions in grey matter and increases in white matter, without change in cerebral

volume (De Bellis, 2001; Paus et al., 2010). Importantly, De Bellis and colleagues (2001) noted that both age-related decreases in grey matter and increases in white matter were significantly more pronounced in males. This finding was replicated in a study conducted in our lab demonstrating greater absolute and relative white matter volumes and slightly lesser relative grey matter volume and lesser grey matter density throughout the cortex in adolescent males, relative to females (Paus et al., 2010). Our lab further tested whether or not testosterone influenced grey matter trajectories in males to account for the observed sex difference, and found this was the case (Paus et al., 2010). Plasma levels of bioavailable testosterone in males correlated positively with total brain volume, absolute, and relative white matter volume, and negatively associated with relative grey matter. Upon confirmation of this hypothesized relationship, Mendelian randomization was used to compare males based on their having a more or less efficient polymorphism of the androgen receptor gene (Sheehan et al., 2008). Testosterone's influence on relative grey and white matter volumes was indeed moderated by individuals' androgen receptor gene (AR) variant; the effect of testosterone was inflated in those with the more efficient AR polymorphism relative to those with the inefficient variant. Research has since offered further support that sex hormones are at least in part determinant of grey matter volumes. Witte and colleagues demonstrated grey matter was positively associated with estradiol and negatively associated with testosterone in young adults, showing regional effects using VBM in a sample of young adults (Witte, Savli, Holik, Kasper, & Lanzenberger, 2010). One study of adolescents controlling for the effects of both age and pubertal status, observed sex differences in the relationship between circulating testosterone and thickness in brain regions with high densities of *CB1* receptors (Brahmen et al., 2012). The additional discovery that some brain regions correlated positively with testosterone indicates that the negative association typically seen is not uniform throughout the cortex (Brahmen et al., 2012). Lastly, one longitudinal study demonstrated this relationship between testosterone and cortical thickness is dynamic and changes over time, evidenced by an age by testosterone interaction on thickness when examining the effect at age between 4 and 22 years old (Ngyugen et al., 2013).

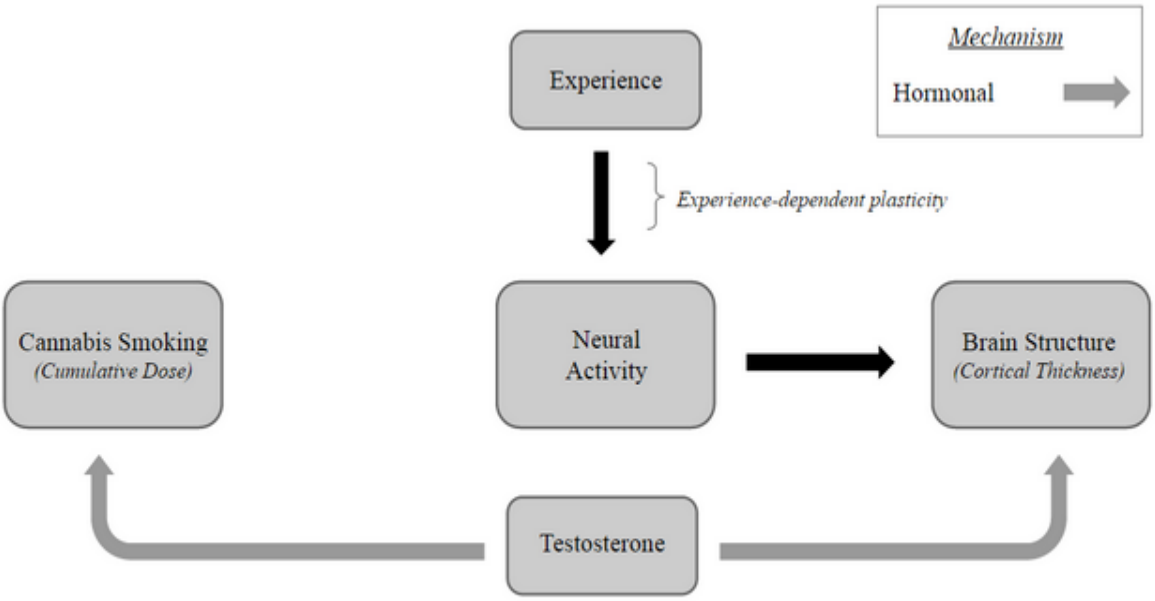
Accompanying the morphological changes associated with puberty is an increase in substance use behaviour. The most common period of drug initiation is adolescence, during which pubertal maturation is taking place. Earlier pubertal onset has been shown to increased likelihood of having used substances (Cance et al., 2013; Kaltiala-Heino, Koivisto, Marttunen, & Fröjd, 2011; Tschann et al., 1994). Students in more advanced pubertal Tanner stages are more than 3 times as likely to report lifetime substance use (including cannabis), as well as cannabis abuse relative to those in earlier stages (Catalano et al., 2004 [Abstract]). Earlier onset puberty was shown to be a risk factor for substance use, particularly among males, predicting lifetime use in a two-year longitudinal study (Kaltiala-Heino et al., 2011). A relatively new body of work headed by Tarter and colleagues has emerged with at least a partial explanation of the relationship between puberty and substance use, pointing to testosterone as a driving factor.

Tarter's research has demonstrated the predictive value of testosterone levels during adolescence vis-a-vis future outcomes in young adulthood. Several publications by this group document the findings throughout a longitudinal study of an at-risk population (Horner et al., 2014; Reynolds et al., 2007; Tarter et al., 2007; 2009; 2013). The study followed children over 10 years into young adulthood, collecting data at four follow-ups. Most recently, Tarter et al. (2013) reported plasma levels of testosterone in boys at age 16 were predictive of whether or not they developed a substance use disorder at follow up at the age of 22 years old. Using path analysis, Tarter and colleagues (2013) have been able to illustrate multiple paths from factors at baseline (between ages 10 to 12), as well as the first follow up (between ages 12 to 14) in predicting cannabis use disorder (CUD) in the final follow up (at age 22. One such path showed that frequency of cannabis use at 19 could be predicted directly from percent (%) of abandoned dwellings in their neighborhoods between 10 and 12 years of age (according to census data). Interestingly, an alternate path was also observed, one depicting multiple steps showing T in early adolescence was predictive of cannabis use frequency at 19 and CUD at 22 vis-a-vis two intermediary factors, or antecedents; specifically, T levels at 10 to 12 were shown predictive of assaultive behaviour at 12 to 14, while T levels at 12 to 14 were shown predictive of both assaultive behaviour at 12 to 14, and social dominance/normative-violating behaviour at 16 - both of which subsequently predicted cannabis use frequency at 19, and cannabis use disorder at 22, respectively (Kirillova, Vanyukov, Kirisci, & Reynolds, 2008; Reynolds et al., 2007; Tarter et al., 2009). Using the same data set with additional data regarding participants' Tanner stages, Horner et al., (2013) performed additional path analyses showing direct predictive paths from pubertal stage at 16 to substance use at 19, and from pubertal stage at 19 to substance use disorder at 22. Tarter his and colleagues' work is highly informative in its further evidencing testosterone as a predictor of later cannabis use, and its elucidation as to the mechanism by which this could occur.

Testosterone has been shown to predict substance use in general, and specifically that of cannabis. Likewise, testosterone has been related in a causal way to reductions in cortical thickness. Thus, we propose testosterone as a possible external factor that could independently cause both low cortical thickness and high CD, resulting in their co-occurrence (see Figure 10. below). This hypothesis could be explored through testing for associations between serum testosterone levels with our dependent and independent variables; namely, a student's t-test comparing mean testosterone of our highest and lowest cumulative dose groups and a calculation of Pearson's correlation coefficient could be used to test this hypothesis. In the event testosterone is associated with both, Mendelian randomization could similarly be used to further explore this hypothesis. If our observed effect is greater in participants with the more efficient polymorphism of the AR gene, it would suggest testosterone is causally related (Sheehan et al. 2008). The number of participants in our Highest CD group would likely not be high enough to test this with sufficient power, though it should be considered in future studies (Paus et al., 2010). Alternatively,

serum testosterone levels could be added to our ANCOVA models, to see if our observed effect survives statistical significance after adjusting for serum testosterone level as a covariate.

Figure 7. Potential indirect mechanism relating cannabis smoking to cortical thickness



4.3 Strengths

Our analyses was benefited by a large number of participants, especially for a study acquiring MRI data, rendering our analyses statistically powerful; post-hoc power calculations our test was shown to have moderate power to detect the tested difference.

A prospective longitudinal design yielded cannabis data across multiple time points, from which we were able to assign participants to comparison groups with confidence, and ensure accuracy in our data. Rather than relying on participant reports of cannabis use onset, we were able to deduce this variable from examination at what age participants first reported using cannabis, minimizing error inherent in self reporting cannabis use retrospectively due to human error. In examining participants' data longitudinally we were able to perform quality control of participant self-report data and exclude those participants who we could not confidently classify.

Characteristics of our sample allow our findings to be highly generalizable. Structural brain differences were demonstrated in a nonclinical sample. Further, our nonclinical sample was not highly specific in that we used all participants, creating cannabis use groups based on the entire spectrum of cannabis use in our sample. Our results thus represent the possible effects of socially relevant levels of cannabis use representative of the general public. This is in contrast to many studies recruiting participants who meet thresholds of cannabis use, which are often arbitrary and outside of the normal range found in the population, and are statistical outliers. Studying the full range and variety of cannabis use in our sample, we imposed no inclusion criteria on participants unlike research with highly specific exclusion criteria. Previous studies of long-term users, chronic users, problem cannabis users, or users seeking treatment whose results are limited in generalizability even among cannabis users. Our participants form a subset of a birth cohort in a population study and thus their levels of cannabis use is representative of that naturally occurring in the population at large. Our results therefore apply to socially relevant levels of cannabis exposure. For example, our highest exposure group had a minimum cutoff of smoking cannabis on 60 occasions, in comparison with participants having smoked between a range of 675 to 10,150 "joints" in a study by Jager and colleagues (2007), and between 4600–288000 in Yucel et al.'s study (2008). Relative to studies showing structural differences in individuals with problematic or chronic cannabis smokers, it is notable that our highest cumulative dose level of "over 60 times" was able to predict group differences.

Operationalization of our cannabis use was also advantageous. Comparison groups were based on binary and as ordinal measures of cannabis use, the latter enabling the comparison of different levels of use. The advantage of an ordinal measure was in our finding cortical thickness was not associated with a binarized measure of cannabis use, but was associated with our ordinal factor. Our finding the difference in thickness was between the two extremes of our ordinal levels may not have been detected if we had collapsed or binarized individual levels of CD. In demonstrating differences in cortical thickness were present in participants who had smoked cannabis a minimum of 61 times, we provide evidence that levels in this range should be considered in the design of future studies. For example, cannabis use approaching levels as low as 60 times are sufficient for exclusion criteria in a control group in studies of cannabis use and brain structure. For example, in a study by Cousjin et al., (2011), the cutoff of cannabis use for the control group was 50 lifetime uses of cannabis, with 5 of 42 subjects having over 10 lifetime uses. Based on our results, these analyses may have been confounded based on their cutoff criteria for a control group. Our results highlight the value in acquiring cumulative estimates of lifetimes use, as well as acquiring multiple metrics of cannabis use, as we saw in our ANCOVA models only one of four cannabis use metrics significantly predicted cortical thickness; ever use, onset, and frequency within the last year all showed insignificant effects. Additionally we recommend future studies collect an estimate of cumulative lifetime exposure to cannabis and to use this data in analyses and compare participants within a spectrum

of cannabis use. Our results suggest future studies move away from comparing cannabis between comparison groups that may be qualitatively different based on an arbitrary cutoff, and towards comparisons based on multiple dimensions and in ordinal terms in conjunction with categorical ones. Benefits of cumulative lifetime measures, and ordinal and scalar metrics of cannabis use could potentially reduce inconsistencies in future research, and possibly even explain previous ones. As we know from our study and previous literature, not all cannabis use appear to elicit detectable changes in brain structure. Studying cannabis use on a spectrum as we have done in this study would yield a higher chance of capturing the minimum threshold of use necessary for the effect to occur.

Another potential strength of our research, which may explain our significant findings compared with previous studies finding none, is that our participants were youth. While the developing adolescent brain may be more vulnerable to the effects of cannabis, we must also consider that alternatively, younger participants' brains may have suffered less structural consequences due to aging, stress, or health and psychiatric conditions, making it easier to detect differences due to cannabis use. Individual differences that could mask or obscure those of cannabis use may be minimized at this early stage in life (within the same geographic location and culture), which could reduce noise in structural data and allow more subtle differences due to lower to moderate levels of cannabis use to be detected.

4.4 Limitations

As mentioned above, this study did not collect brain data at multiple time points - in light of this limitation we cannot speak to causation, nor are we able to infer directionality of hypothetical changes. For instance, any group discrepancies in cortical thickness could have predated initiation of substance use. This alternative hypothesis is not unfounded given previous research findings that individual differences in brain structure may in fact predisposed individuals to substance use. Research of the first of its kind was conducted by Cheetham and colleagues in 2012, showing with longitudinal data that reduced volume of the orbitofrontal cortex at 12 years old not only preceded future substance use, but was actually predictive of future substance use at 16 years old. This study was unique in its testing of whether brain structure could predict drug use, and serves to highlight our need to consider non causal hypotheses alongside the hypothesis that CD caused cortical thickness, given evidence the reverse could be true.

Since the subset of participants for whom cumulative dose data were collected and analyzed were all early onset cannabis users, we cannot say whether this relationship holds true when cannabis smoking is initiated in later adolescence, after the age of 16.5.

Additionally, CD symptomatology was not assessed by a trained clinician, but was instead calculated as a percent chance the child at 10 years old would be diagnosed with CD as calculated by a computer algorithm based on ICD-10 and DSM-IV symptom criteria. This was based on parent reports of behaviours exhibited by their children, and was therefore potentially affected by factors such as bias about

presenting their child in a certain way, or the amount of time the parent spent with the child. Therefore, it is possible that CD symptoms were over or under-estimated for some of our participants.

In terms of the interpretation and generalizability of our results, our greatest limitations lie in our self-report measures on which our independent variables and the majority of our confounders are based. Data were self-reported by adolescents or in the case of maternal social class and participants' CD symptoms at age 10, by their parents. Knowing that self-report data are – by definition - subjective and therefore vulnerable to human error, it is possible that estimates of substance use are not entirely accurate. We took care, however, to exclude questionable self-reporting through quality control, as discussed in a previous section. Given the sensitive nature of illicit drug use, it is possible that substance use was falsely reported as negative when in fact positive, or underreported in terms of cumulative dose. While having its disadvantages, self-report was beneficial overall and was the best option possible for obtaining measures of this sort. Through self-reports we have access to a certain degree of accuracy we would not else be able to achieve if for instance another individual was to estimate someone else's substance use. For example parents and peers may or may not be aware of the extent of an adolescent's substance use, or that an adolescent engages in substance use at all. This proved to be the case in our sample since parental knowledge of any drug use was collected multiple times throughout the study. We found large discrepancies between parents' estimates and their children's self-reporting, with parents tending to under report cannabis experimentation of their children.

How we chose to operationalize our Cannabis Use (CU) variable likely limited our ability to detect differences and may explain inconsistency between our findings with some of those in the literature. Namely, Lopez-Larson et al. (2011), Mata et al. (2010), and Matochik, Eldreth, Cadet, and Bolla (2005) found cannabis users exhibited both decreased and increased thickness in various cortical regions, thinner cortical thickness in the superior prefrontal cortex, and lower grey matter density in the right parahippocampal gyrus paired with greater grey matter density in the right thalamus and bilateral precentral gyrus, respectively. Likewise, onset of cannabis use by the age of 17 has been shown to differentiate structural brain measures. Using MRI, Wilson et al., (2000) reported early onset users to have lower whole brain and percent cortical gray matter, while Lopez-Larson et al. (2011) found age of onset negatively correlated with cortical thickness of of the right superior frontal gyrus in adolescent heavy cannabis users. In contrast, our CU variable incorporating onset of cannabis use showed almost no differences with the exception of just reaching significance in our final ANCOVA model did this variable just reach significance. Recall from our described methodology above, we compared participants who had never once used cannabis (Never Users) with those who had used cannabis ever once before (Early Users) or after (Late Users) the age of 16.5. This operationalization was based on the goal of comparing the maximum number of participants based on their cannabis use/nonuse, and was limited based on the wording of the question asking participants if they had ever used cannabis. Regarding this lack of

differences exhibited by CU use groups, we hypothesize our threshold of categorizing early and late cannabis users based on having ever once smoked cannabis was too low for there to be detectable differences. This notion is supported by our observing significant differences between CD groups, suggesting smoking cannabis potentially leads to structural differences after at least 60 occasions of smoking cannabis. Regarding an effect of cannabis onset, we consider our CU variable to have acted as a much less robust version of our CD variable. This is supported by our finding significant differences in lifetime cannabis dose in young adulthood in our sample (with Early Onset users having a higher cumulative lifetime dose). Further, since our CU variable yielded no differences based on cannabis usage despite differences based on our CD variable evidencing the contrary, we suggest our CU variable likewise was not sufficiently robust to detect differences. We therefore suggest we do not have sufficient evidence to conclude whether or not there is likely an effect of cannabis use onset on brain structure and whether we our CD variable would produce similar findings in late onset users.

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